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Research Article

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Mechanism of Activation of Coagulation Factor XI by Factor XIIa Studied with Monoclonal Antibodies

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Abstract

The interaction of Factor XIIa with Factor XI was investigated using two monoclonal antibodies, one (3C1) directed against the heavy chain of Factor XIa and the other (5F4) against its light chain. 3C1 either as intact IgG or as Fab' fragment, enhanced the rate of Factor XIa generation in the fluid phase but inhibited it in the presence of kaolin and high molecular weight (HMW) kininogen. In contrast, the Fab' fragments of 5F4 inhibited only the fluid phase activation and had no effect on the surface-mediated activation. 3C1 was found to block the binding of Factor XI to HMW kininogen, whereas 5F4 did not. We conclude: (a) a domain on the heavy chain region of Factor XI is essential for binding to HMW kininogen and for optimal surface-mediated activation by Factor XIIa; and (b) binding of 3C1 to Factor XI changes its conformation rendering it a more favorable substrate for Factor XIIa in the fluid phase.

Introduction

Factor XI is a blood coagulation protein that participates in the early or contact phase of blood coagulation. Contact activation involves a sequence of events that is known to initiate intrinsic blood coagulation (1, 2), fibrinolysis (3), and the kinin-forming pathway (4). Three other proteins in addition to Factor XI that participate in the contact phase of coagulation are Factor XII, prekallikrein, and high molecular weight (HMW)¹ kininogen. Although it is known that negatively charged surfaces accelerate the activation of prekallikrein by Factor XIIa and conversely the activation of Factor XII by kallikrein (5), the mechanism by which contact activation is initiated is still an open question and its significance in vivo has yet to be established. Interestingly, whereas a deficiency of Factor XI can result in excessive bleeding after trauma or minor surgery (6, 7), deficiencies of any of the other three proteins, namely Factor XII, prekallikrein, or HMW kininogen, are not accompanied by a bleeding disorder. Furthermore, the Factor XIa-catalyzed activation of Factor IX is

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1. Abbreviations used in this paper: HMW, high molecular weight; PAGE, polyacrylamide gel electrophoresis.

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a kinetically favorable reaction (8-10) leading to the generation of thrombin and the formation of fibrin. To understand more fully the pivotal role of Factor XI in coagulation, further studies regarding the mechanisms of activation of the proteins involved in contact activation are necessary.

The present study was undertaken to gain an insight into the mechanism of activation of Factor XI by Factor XIIa both in the fluid phase as well as in the presence of negatively-charged surfaces and HMW kininogen. Human Factor XI consists of two identical polypeptide chains held together by disulfide bond(s) and has an apparent molecular weight of 160,000 (11). During activation by Factor XIIa an internal peptide bond in each of the two 80,000-mol-wt chains is cleaved resulting in the formation of a pair of disulfide-linked heavy and light chains with molecular weights of 50,000 and 30,000 respectively (11-13), with each light chain containing one active site. Factor XI circulates in plasma noncovalently complexed with HMW kininogen (14-16). In the presence of negatively charged surfaces, such as glass, kaolin, or sulfatides, these proteins form a ternary complex with Factor XIIa (5). Complex formation on a surface results in an acceleration of the rate of activation of Factor XI by Factor XIIa, compared with the fluid phase reaction (17).

This paper describes studies of the activation of Factor XI by Factor XIIa both in the fluid phase and in the presence of negatively charged surfaces. Experiments with monoclonal antibodies against different epitopes of Factor XI, used as structure-function probes, reveal that domains in both the heavy chain and the light chain regions are essential for the efficient activation of Factor XI by Factor XIIa.

Methods

Materials. Factor XI was purified from human plasma by a modification (18) of the method of Bouma and Griffin (12). It appeared as a single band at an apparent M_r of 160,000 on a nonreduced SDS-polyacrylamide gel and as a single 80,000-mol-wt band on reduced gels and had a specific activity of 270 U/mg protein as previously reported (18). HMW kininogen was purified by the method of Kerbiriou and Griffin (19). Corn trypsin inhibitor, a specific inhibitor of Factor XIIa (20), was purified as described previously (21) and insolubilized on CNBr-activated agarose (22). Bovine Factor XII was purified by a modification of a previously published procedure (21). 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 M Tris and 2 M sodium thiocyanate (pH 8.0). It was then dialyzed to remove the thiocyanate. This preparation demonstrated a single band of 78,000 mol wt on non-reduced SDS-polyacrylamide gels and two bands of apparent M_r of 52,000 and 28,000 after reduction with dithiothreitol. Factor XIIa concentration in solutions was routinely estimated from its amidolytic activity. Factor XIIa assays were performed in 0.5 ml of 0.1 M Tris buffer (pH 8.0) containing 0.1 mM S-2302, at 37°C. Under these conditions pure Factor XIIa produced a $\Delta OD_{405}/\text{min}$ of 0.08 per microgram. The chromogenic substrate pyro-Glu-Pro-Arg-pNA (S-2366) was kindly given by Dr. Petter Friberger (AB KABI Peptide Research, Molndal, Sweden) and H-D-Pro-

Phe-Arg-pNA (S-2302) was purchased from AB KABI Peptide Research. Benzamidine hydrochloride, polybrene (hexadimethrine bromide), crystallized bovine serum albumin (BSA), and cephalin (rabbit brain extract) were purchased from Sigma Chemical Co., St. Louis, MO. Carrier-free Na ¹²⁵I was obtained from New England Nuclear (Boston, MA). Acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethyl ethylene diamine, Biogel A-1.5 m, and SDS were purchased from Bio-Rad Laboratories (Richmond, CA). Plasmas deficient in Factor XI or HMW kininogen were obtained from George King Biomedical (Overland Park, KA).

Monoclonal antibodies. Details of the production and purification of monoclonal antibodies against Factor XI have been previously published (23). Briefly, IgG fractions from the ascites fluids were isolated by ammonium sulfate precipitation followed by gel filtration using BioGel A-1.5 m. The monoclonal antibodies were further purified to apparent homogeneity by fast protein liquid chromatography using a Mono Q column, according to instructions provided by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ). The monoclonal antibodies used in the present study appeared as pure heavy and light chains of IgG by SDS polyacrylamide gel electrophoresis (PAGE). They were devoid of demonstrable protease activity, i.e., they did not cleave either Factor XI or Factor XIIa, and did not affect the amidolytic activity of either Factor XIa or Factor XIIa. Chain typing of monoclonal antibodies by immunodiffusion using rabbit antimouse antibodies (Miles Laboratories, Inc., Elkhart, IN) revealed that both murine monoclonal antibodies (5F4 and 3C1) were class IgG_{1κL}. Neither of the monoclonal antibodies cross-reacted with either Factor XII or HMW kininogen, because neither of those coagulation proteins was bound to monoclonal antibody affinity columns, whereas Factor XI was (23), because the activities of these two proteins were unaffected when the antibodies were incubated with human plasma, whereas Factor XI activity was inhibited (23).

Coagulation and amidolytic assays. Factor XI and HMW kininogen were assayed by minor modifications (24) of the kaolin-activated partial thromboplastin time (25) using appropriate congenitally deficient substrate plasmas, and results were quantitated on double logarithmic plots of clotting times versus concentrations of pooled normal plasma. Factor XIIa was assayed using the chromogenic substrate S-2302 according to the procedure described previously (21). The amidolytic assay of Factor XIa was carried out by a modification of the method of Scott et al. (24). Incubation mixtures described in detail in the figure legends were assayed for Factor XIa as follows: 10 μl of the reaction mixture containing Factor XIa was transferred to a cuvette containing 250 μl of phosphate buffer (0.1 M)/NaCl (0.15 M), (pH 7.6; PBS) made 1 mM in EDTA and 0.66 mM in S-2366. The rate of hydrolysis was recorded at 405 nm using a spectrophotometer (model 2600; Gilford Instrument Laboratories, Inc., Oberlin, OH). Factor XIa was quantitated from a standard curve prepared using purified Factor XIa. The background activity was measured in all experiments using a similar incubation mixture containing all the components except Factor XIIa and background values were subtracted from all data points. Factor XIIa by itself at the concentrations used did not cause significant hydrolysis of the chromogenic substrate.

Protein analyses. Protein concentrations were determined by the BioRad dye binding assay using BSA as a standard (26). Purified monoclonal antibodies were quantitated using an extinction coefficient of 14 for a 1% solution at 280 nm. Polyacrylamide slab gel electrophoresis in SDS was performed by the procedure of Laemmli (27). Gels were stained, dried onto paper, and autoradiograms were prepared from the dried gels using intensifier screens (DuPont Cronex Lighting-Plus Screens, mounted in Spectrolin cassettes, Reliance X-Ray Inc., Orelan, PA). Kodak X-Omat-AR film was used and developed according to instructions provided with the film. To quantitate percent cleavage of ¹²⁵I-labeled Factor XI, the dried gels were cut into lanes, which in turn were cut into transverse strips. By counting the radioactivity in the individual strips and subtracting background counts, the amount of radioactivity in the 80,000-mol-wt Factor XI zymogen and the amount in cleavage products (50,000 and 30,000 mol-wt) representing Factor XI was determined. Percent cleavage was calculated by dividing the amount of radioactivity in cleavage products by the total amount of radioactivity in the lane examined.

Radiolabeling of proteins. Purified Factor XI was radiolabeled by a minor modification (18) of the Iodogen method (28) to a specific activity of 5×10^6 cpm/μg. The radiolabeled protein retained >90% of its biological activity.

Binding of Factor XI to HMW kininogen. Effects of the antibodies on the binding of Factor XI to HMW kininogen were studied using polyvinyl chloride microtiter plates, the wells of which were coated with HMW kininogen by incubation with 100 μl of the protein (100 μg/ml) for 2 h at room temperature. Residual binding sites on the wells were blocked by incubating with 200 μl of 5 mg/ml BSA in PBS (PBS/BSA). After washing the wells with PBS/BSA to remove any unbound HMW kininogen, 100 μl of a mixture of ¹²⁵I-Factor XI (6 μg/ml) and either buffer or antibody (preincubated for 30 min at room temperature in polypropylene tubes precoated with 5 mg/ml BSA) were added to the wells and incubated for 3–4 h at room temperature. ¹²⁵I-Factor XI/buffer incubation mixture was also added to additional wells that were coated with BSA only without precoating with HMW kininogen to determine background counts arising from binding of ¹²⁵I-Factor XI to BSA. The wells were thoroughly washed with PBS/BSA, dried, and counted in a gamma counter.

Preparation of Fab' fragments of antibodies. Fab' fragments of the monoclonal antibodies to Factor XI were prepared as follows: 8 mg of the purified antibody (IgG) in 1 ml of acetate (0.1 M, pH 4.5) was incubated with 160 μg of pepsin for 24 h at 37°C. The reaction was stopped by raising the pH to 8.0 using saturated Tris-base solution. By this procedure > 85% of the IgG was digested to F(ab')₂ as judged by SDS-PAGE. To prepare Fab' from F(ab')₂, mercaptoethanol at a final concentration of 10 mM was added to the reaction mixture and incubated for 2 h under N₂ in the dark at room temperature. The reduced fragments were then alkylated in the presence of 50 mM iodoacetamide under the same conditions as used for reduction. The reduced and alkylated material was dialyzed against 0.02 M phosphate and 0.15 M NaCl at pH 7.4 and purified using a protein A Sepharose column. This preparation contained 80–85% Fab' (*M_r* ~ 55,000). Two other bands comprising 15–20% of the preparation (*M_r* ~ 22,000 and ~25,000 respectively) were the products of reduction of disulfide bonds between heavy and light chains.

Results

Activation of Factor XI by Factor XIIa in the fluid phase. We have previously reported on the production, characterization, and use of murine hybridoma antibodies directed against various epitopes in human coagulation Factor XI (23). One of these monoclonal antibodies (5F4) is directed against a region in the light chain of Factor XIa distinct from the active site, because it binds to the reduced and alkylated light chain of Factor XIa. The binding region is distinct from the active site, however, since this antibody significantly inhibits Factor IX activation by Factor XIa without affecting the amidolytic activity of Factor XIa (23). Another monoclonal antibody (3C1) has been shown to bind the heavy chain of reduced and alkylated Factor XIa without affecting the amidolytic activity of intact Factor XIa (23). We have now used these two antibodies as structure-function probes to examine the mechanism of activation of Factor XI by Factor XIIa. After incubation of Factor XI (containing trace ¹²⁵I-labeled Factor XI) with monoclonal antibodies or with buffer, Factor XIIa was added and the rate of generation of Factor XIa was measured.

Fig. 1 shows the effects of the two antibodies on the proteolytic cleavage of Factor XI by Factor XIIa, which is representative of four similar experiments. Autoradiograms of the SDS-PAGE of progress curves of control and antibody-treated samples are shown. Enhancement of the rate of proteolytic cleavage of Factor XI by the heavy chain-specific antibody 3C1 and its inhibition by the light chain-specific antibody 5F4 compared with control samples is apparent from the results (Fig. 1). When Factor XI

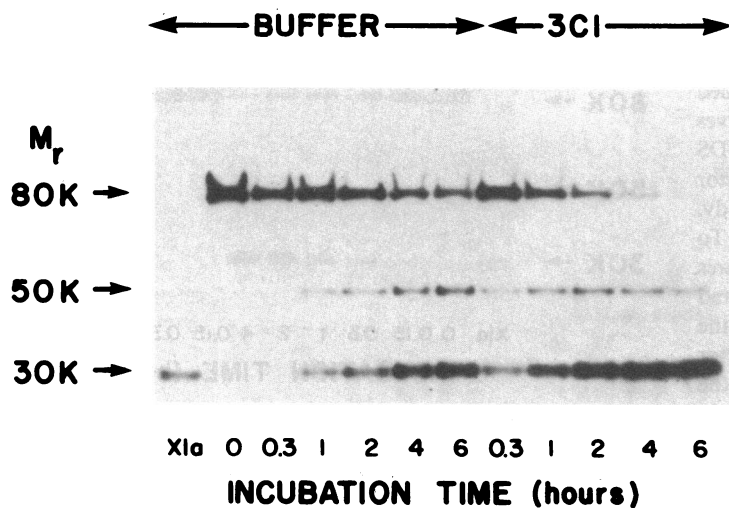
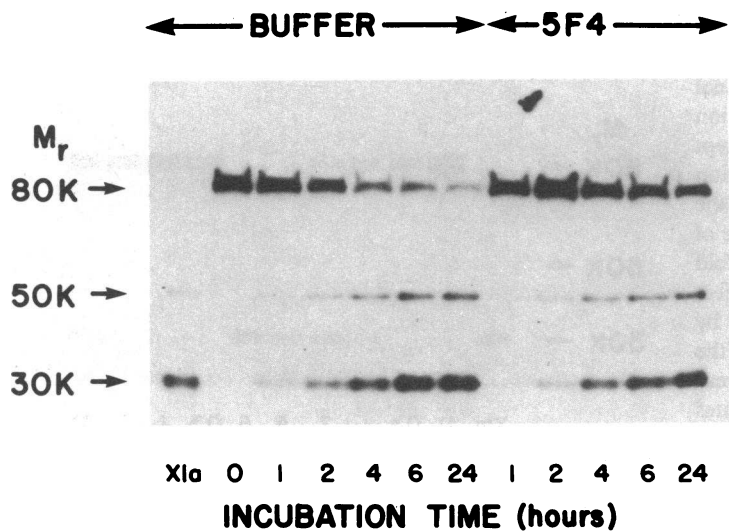


Figure 1. Effects of monoclonal antibodies against the heavy chain (3C1) or the light chain (5F4) of Factor XI on the proteolytic cleavage of Factor XI by Factor XIIa in the fluid phase. Factor XI ($0.17 \mu\text{M}$) containing trace ^{125}I -Factor XI was incubated with either PBS or IgG solution ($2.5 \mu\text{M}$) for 10 min at 37°C in the presence of BSA (1 mg/ml). Factor XIIa ($0.017 \mu\text{M}$) was then added and the reaction mixture was incubated at 37°C . At different intervals, aliquots were removed either into buffer containing corn trypsin inhibitor ($0.034 \mu\text{M}$) for measuring Factor XIa generated (see Fig. 2) or into buffer containing SDS to examine the cleavage products by SDS PAGE (8%) in the presence of 2-mercaptoethanol and radioautography, as described in Methods. Results shown are those with PBS, which were similar to those with control IgG (data not shown); with the heavy chain-specific monoclonal antibody, 3C1; and with the light chain-specific antibody, 5F4.

activation was determined by the chromogenic assay for Factor XIa (Fig. 2 A) it was apparent that a sixfold increase in the initial rate of Factor XI activation occurred in the presence of the heavy chain-specific antibody, 3C1, and this rate enhancement was confirmed by measurement of rates of proteolytic cleavage of Factor XI determined by counting the radioactivity in gel slices (Fig. 2 B). By comparison, it is apparent that the rate of proteolytic activation of Factor XI was decreased to 30–50% of control values in the presence of the light chain specific antibody, 5F4 (Fig. 2, A and B). Control experiments with both monoclonal antibodies, 5F4 and 3C1, demonstrated no amidolytic activity against the chromogenic substrates S-2366 or S-2302 and no effect of either antibody on the amidolytic activity of Factor XIa, using S-2366 as substrate (data not shown). In addition, control experiments were carried out with murine IgG at concentrations similar to those of monoclonal antibodies. Because results similar to those obtained with control buffer solutions were obtained, detailed control data are reported for buffer controls. It should be emphasized that in these experiments and all those reported below, the concentrations of antibodies used were in excess of saturating concentrations.

Activation of Factor XI by Factor XIIa in the presence of a negatively charged surface. Because negatively charged surfaces

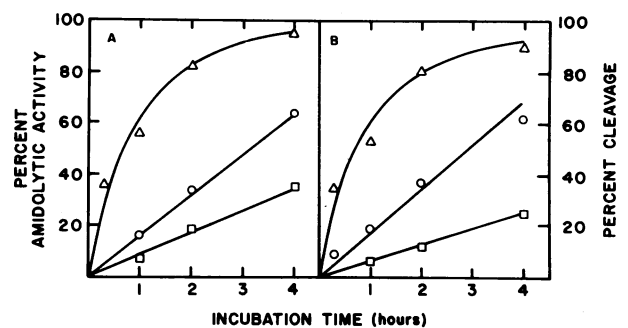


Figure 2. Effects of monoclonal antibodies against the heavy chain (3C1) or the light chain (5F4) on the activation of ^{125}I -Factor XI by Factor XIIa in the fluid phase. The details of the experiment are presented in the legend to Fig. 1. Amidolytic assay was performed according to the procedure described in Methods, and A shows the results. Percent cleavage of Factor XI was determined by cutting and counting the radioactivity in SDS gels as described in Methods, and B shows the results. Results shown are those obtained with PBS (open circles), which were similar to those obtained with control IgG (data not shown); with the light chain-specific monoclonal antibody, 5F4 (open squares); and with the heavy chain-specific antibody, 3C1 (open triangles).

such as kaolin, sulfatides, or dextran sulfate are known to enhance the rate of activation of Factor XI by Factor XIIa (17) it was of interest to examine the effects of the same monoclonal antibodies on the rate of this reaction. The rate of the activation was studied essentially the same way as in the fluid phase except that HMW kininogen and kaolin were included in the reaction mixture. Fig. 3 illustrates the enhancement of the rate of the activation by the negatively charged surface. In the presence of kaolin the initial rate was enhanced by about five to six fold compared with fluid phase. We have studied in detail the effect of HMW kininogen on the rate of activation of Factor XI by Factor XIIa at various concentrations of kaolin as well as in the absence of a surface. It was observed consistently that whereas the presence of HMW kininogen is essential for optimal rates of kaolin-mediated activation, it was not required and did not influence the rate of Factor XI activation in the absence of a contact-activating surface (data not shown).

Because we were interested in studying the functions of different domains of Factor XI in the surface-mediated activation of Factor XI, we focused our attention on the effects of monoclonal antibodies in the presence of kaolin and HMW kininogen. Factor XI was preincubated with HMW kininogen and antibody solution or buffer for 10 min at 37°C and subsequently added to a mixture of kaolin and Factor XIIa. When progress curves of these mixtures were examined by autoradiography of SDS gels it was apparent that the rate of proteolytic cleavage of Factor XI was inhibited by both the heavy chain-specific antibody, 3C1, and the light chain-specific antibody, 5F4 (Fig. 4). To quantitate the effects of these antibodies, the incubation mixtures were examined for Factor XIa amidolytic activity (Fig. 5 A) and for the extent of proteolytic cleavage of Factor XI by slicing the SDS gels and determining the amount of radioactivity in Factor XIa cleavage products (Fig. 5 B). Fig. 5 A demonstrates that the initial rate of Factor XI activation was decreased by >80% in the presence of the light chain-specific antibody (5F4) and by 70% in the presence of the heavy chain-specific antibody (3C1). Similar results were obtained when the proteolytic cleavage of Factor XI was examined by 8% PAGE in the presence of SDS and 2-mercaptoethanol (Fig. 5 B). These results, which are representative of three similar experiments, suggest that domains on both the heavy chain and the light chain regions of Factor

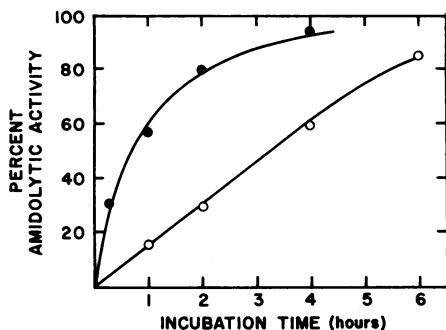


Figure 3. Activation of Factor XI by Factor XIIa in the fluid phase and in the presence of negatively charged surfaces. The experiment was carried out as described in the legend to Fig. 1 except that the final incubation mixture consisted of 0.17 μ M Factor XI containing a trace amount of 125 I-Factor XI, 0.07 μ M HMW kininogen, 1 mg/ml BSA, 0.017 μ M Factor XIIa, and either 0.5 mg/ml kaolin (filled circles), or PBS buffer (open circles). Factor XIa amidolytic activity was assayed as described in Methods.

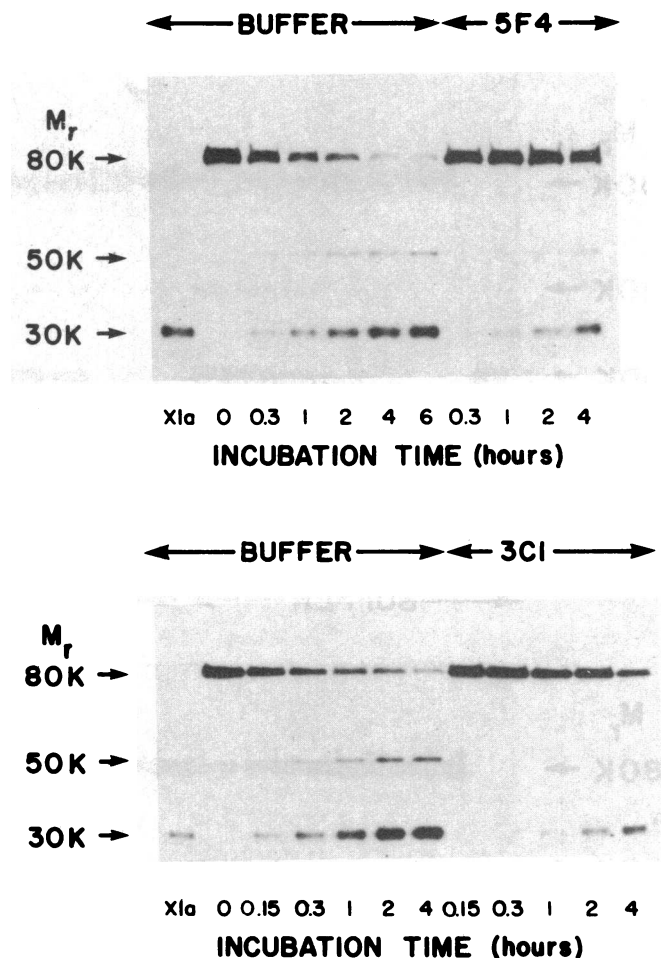


Figure 4. Effects of monoclonal antibodies against the heavy chain (3C1) or the light chain (5F4) on the proteolytic cleavage of Factor XI by Factor XIIa in the presence of kaolin and HMW kininogen. Factor XI (0.17 μ M) containing a trace amount of 125 I-Factor XI was preincubated with HMW kininogen (0.07 μ M), BSA (1 mg/ml), and IgG solution (2.5 μ M) or PBS buffer for 10 min at 37°C. This mixture was then added to a mixture of kaolin (0.5 mg/ml), Factor XIIa (0.017 μ M) and BSA (1 mg/ml). At the times indicated aliquots were removed into buffer containing corn trypsin inhibitor and Factor XIa amidolytic activity was measured (See Fig. 5) or into SDS for 8% PAGE and autoradiography as described in Methods. Results shown are those with buffer, heavy chain-specific antibody 3C1, and light chain-specific antibody 5F4.

XI are involved in the activation of Factor XI by Factor XIIa in the presence of HMW kininogen and kaolin.

Effects of Fab' fragments of the monoclonal antibodies on the activation of Factor XI by Factor XIIa. The enhancement of the rate of activation of Factor XI by Factor XIIa in the presence of the heavy chain-specific antibody (3C1) is most likely the result of a conformational change in the Factor XI molecule rendering it a more favorable substrate for cleavage by Factor XIIa. However, the inhibitory effects of the light chain-specific antibody (5F4) both in the fluid phase as well as in the presence of a surface and the inhibitory effects of the heavy chain-specific antibody (3C1) in the presence of kaolin and HMW kininogen could have the following alternative explanations: (a) the antibodies are blocking the cleavage site of Factor XI; (b) the antibodies are blocking the interaction of Factor XIIa with a site

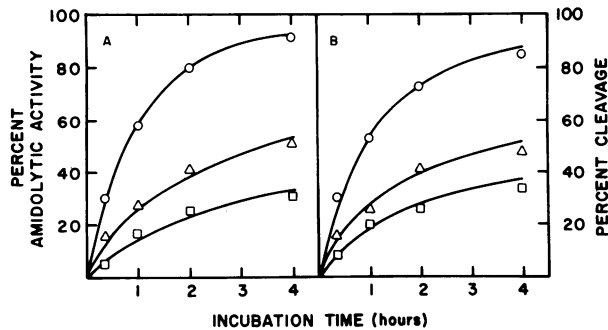


Figure 5. Effects of monoclonal antibodies against the heavy chain (3C1) or the light chain (5F4) on the activation of Factor XI by Factor XIIa in the presence of kaolin and HMW kininogen. The details of the experiment are presented in the legend to Fig. 4. Amidolytic assay was performed as described in Methods, and results are shown in *A*. Percent cleavage of Factor XI was determined by cutting and counting the radioactivity in SDS gels as described in Methods. Results are shown in *B*. Data shown are those obtained with PBS (*open circles*), which were similar to those obtained with control IgG (data not shown); with the light chain-specific antibody, 5F4 (*open squares*); and the heavy chain-specific antibody, 3C1 (*open triangles*).

other than the cleavage site on the light chain or the heavy chain of Factor XI required for activation; or (c) in the case of the surface reaction, the heavy chain-specific antibody (3C1) interferes with the binding of Factor XI through HMW kininogen to the surface. To determine whether the cleavage site is sterically hindered by these antibodies, the rate of activation was studied in the presence of the Fab' fragments. The results are shown in Fig. 6. Fab' fragments of 3C1 showed similar effects compared with the intact antibody both in the fluid phase (Fig. 6 *A*) as well as in the presence of the surface (Fig. 6 *B*). However, Fab' fragments of the light chain-specific antibody (5F4), although they inhibited fluid phase activation (Fig. 7 *A*) similar to intact antibody (Figs. 1 and 2), had no effect on the rate of Factor XI activation by Factor XIIa in the presence of kaolin and HMW kininogen (Fig. 6), whereas the intact monoclonal antibody (5F4) was inhibitory (Figs. 4 and 5).

Effects of monoclonal antibodies on the binding of Factor XI to HMW kininogen. Previous studies suggest that the heavy chain region of Factor XI contains binding sites for HMW kininogen (29). Because the heavy chain specific monoclonal antibody (3C1) significantly inhibited the rate of Factor XI activation by Factor XIIa in the presence of HMW kininogen and kaolin (Figs. 4 and 5), we postulated that the heavy chain-specific antibody (3C1) might be interfering with the binding of Factor XI to HMW kininogen and thereby preventing surface assembly of the Factor XI-HMW kininogen complex for efficient activation by Factor XIIa. To explore this possibility, the effects of our monoclonal antibodies on the binding of Factor XI to HMW kininogen were examined. Using ^{125}I -Factor XI we first attempted to study the effects of the antibodies on the binding of Factor XI to kaolin through HMW kininogen. However, we found that even in the absence of HMW kininogen 30–40% of Factor XI binds to kaolin and this could not be prevented even in the presence of BSA (20 mg/ml). It is quite possible, therefore, that in the presence of HMW kininogen, binding of Factor XI to kaolin occurs both directly as well as through HMW kininogen. In the absence of detailed knowledge of these two separate equilibrium processes, interpretation of the effects of the antibodies became extremely

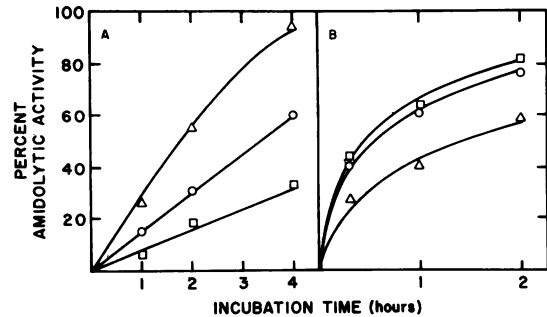


Figure 6. Effects of Fab' fragments of the monoclonal antibodies on the activation of Factor XI by Factor XIIa. The experiment was carried out with unlabeled Factor XI exactly as described in the legend to Figs. 1 and 4 except that Fab' fragments of the monoclonal antibodies were substituted for intact antibodies and were used at the same molar concentrations as noted for Figs. 1 and 4. Data shown in *A* are for fluid phase activation (i.e., in the absence of kaolin and HMW kininogen). *B*, data collected in the presence of kaolin and HMW kininogen. Results shown are those for additions of PBS (*open circles*); the heavy chain-specific antibody, 3C1 (*open triangles*); and the light chain-specific antibody, 5F4 (*open squares*).

difficult. We therefore chose a different procedure to study the effects of the antibodies on the binding of Factor XI to HMW kininogen. HMW kininogen was bound to the wells of polyvinyl chloride microtiter plates, and after blocking residual sites with BSA, ^{125}I -Factor XI preincubated with either buffer or antibody was added to the wells. Details of the procedure are given in Methods. Under these conditions the binding of ^{125}I -Factor XI to microtiter plates containing no bound HMW kininogen was negligible in the presence of 5 mg/ml BSA (see Table I). Specifically, when the microtiter wells were coated with BSA alone without precoating with HMW kininogen, <1.5 ng of ^{125}I -labeled Factor XI was bound to the wells, whereas when wells were coated with HMW kininogen, 51 ng of ^{125}I -labeled Factor XI were bound. Table I gives the amounts of Factor XI bound to HMW kininogen in the presence and absence of the antibodies.

Table I. Effects of Monoclonal Antibodies on the Binding of ^{125}I -Labeled Factor XI to HMW Kininogen

Concentration of antibody <i>μg/ml</i>	Factor XI bound in presence of:	
	5F4 <i>ng</i>	3C1 <i>ng</i>
0	51.0	51.0
13.3	73.6	16.5
33.3	87.9	10.3
132.0	91.4	5.3
333.0	55.6	2.6

The binding of ^{125}I -labeled Factor XI to HMW kininogen bound to the wells of microtiter plates in the presence of BSA (5 mg/ml) was studied. Details of the experiment are given in the Methods and Results sections. When HMW kininogen was not bound to the wells of the microtiter plate, the amount of ^{125}I -labeled Factor XI bound was <1.5 ng, i.e., <3% of the control value. The maximum variation of CPM bound for each experimental observation was <2% of total CPM bound.

In the case of the heavy chain-specific antibody, 3C1, there was a progressive decrease in the binding of Factor XI to HMW kininogen with increased concentrations of the antibody, whereas control murine IgG had no effect on binding (data not shown). In contrast, the light chain-specific antibody, 5F4, did not inhibit the binding at any of the concentrations studied. In fact, a significant (80%) increase in the binding of Factor XI to HMW kininogen was observed, an effect that most likely arises from the bivalent nature of the antibody. Thus, we suggest that antibody 5F4 binds to the light chain of ^{125}I -labeled Factor XI bound through its heavy chain to HMW kininogen, and then additional ^{125}I -Factor XI molecules are bound to the immobilized antibody. In support of this explanation are the facts that: (a) Fab' fragments of antibody 5F4 did not increase the binding of ^{125}I -Factor XI to HMW kininogen in this experiment (data not shown); and (b) at high concentration of antibody 5F4 (333 $\mu\text{g/ml}$) the increased binding was abolished (Table I) possibly because the fluid phase antibody competes with antibody bound to immobilized Factor XI, thereby effectively sequestering additional Factor XI molecules in solution by saturating fluid phase Factor XI with light chain-specific antibody. Whatever the explanation, however, it is clear that only the heavy chain-specific antibody inhibits the interaction of Factor XI and HMW kininogen, most likely because it recognizes an epitope in the heavy chain close to the HMW kininogen-binding site.

Discussion

Negatively charged substances have been reported to accelerate the rate of activation of Factor XI by Factor XIIa in the presence of HMW kininogen (17). The mechanism by which a negatively charged surface and HMW kininogen facilitate the interaction of Factor XIIa with its substrate Factor XI is not clearly known. The present study was undertaken to gain an insight into the nature of interaction between Factor XI and Factor XIIa during activation and to determine whether domains present on the heavy chain or the light chain or both are involved in the fluid phase and surface-mediated reactions. We have utilized two monoclonal antibodies directed against different epitopes of Factor XI as structure-function probes for this purpose. Figs. 1 and 2 illustrate the effects of the two antibodies on the activation of Factor XI by Factor XIIa in the fluid phase. Because the heavy chain-specific antibody 3C1 enhances the rate of activation of Factor XI in the fluid phase, it is reasonable to assume that in the fluid phase the heavy chain region of Factor XI (or at least the domain on the heavy chain region recognized by 3C1) is not essential for interaction with Factor XIIa. The enhancement by 3C1 of the Factor XIIa-catalyzed activation of Factor XI can be rationalized as due to an alteration in conformation of the Factor XI molecule when 3C1 binds to the heavy chain to make it a better substrate for Factor XIIa. That the rate of fluid phase activation of Factor XI by Factor XIIa in the presence of the 3C1 antibody (Figs. 1 and 2) was similar to that occurring without antibody in the presence of kaolin and HMW kininogen (Figs. 4 and 5), indicates that the heavy chain-specific antibody can substitute for both HMW kininogen and kaolin.

HMW kininogen did not have any effect on the activation of Factor XI by Factor XIIa when the reaction was carried out in the fluid phase (Fig. 3 and Results), in agreement with results previously reported (17). In contrast, it had a major effect when the activation was carried out in the presence of kaolin. Factor XI is known to circulate in plasma in a noncovalent complex

with HMW kininogen (14-16), and since in the presence of a negatively charged surface, HMW kininogen enhances the rate of activation of Factor XI by Factor XIIa, it has been postulated that the three proteins form a ternary complex on the surface during contact activation (5). It is not known exactly how these three proteins interact with each other. If it is essential that Factor XI binds to the surface via HMW kininogen for its efficient cleavage by Factor XIIa, then we should see an inhibition of the rate of activation if its binding to HMW kininogen is somehow blocked. The observation (29) that the isolated heavy chain of Factor XIa binds to HMW kininogen whereas the isolated light chain does not suggests that the binding site on Factor XI for HMW kininogen is located on the heavy chain region. However, it is possible that the proteolytic cleavage, reduction, and alkylation of Factor XI required for these studies (29) might alter the binding capacities of the isolated polypeptide chains. Therefore, we chose to investigate this question using intact native Factor XI and monoclonal antibodies directed against chain-specific epitopes. Our data (Table I) demonstrate that while the light chain-specific antibody 5F4 does not inhibit the binding of HMW kininogen to Factor XI, the heavy chain specific antibody 3C1 does. Figs. 4 and 5 show that the same antibody also inhibits the rate of activation of Factor XI by Factor XIIa in the presence of kaolin and HMW kininogen. This inhibition can, therefore, be attributed to prevention of complex formation between HMW kininogen and the heavy chain region of Factor XI.

The light chain-specific antibody 5F4 inhibited the rate of activation both in the fluid phase as well as in the presence of the negatively charged surface and HMW kininogen. This inhibition suggests an interaction between the light chain region of Factor XI and Factor XIIa, which is essential for efficient activation. However, it does not rule out the possibility of a steric inhibition caused by the binding of the antibody as a result of which Factor XIIa cannot gain access to the cleavage site. To investigate this possibility, experiments were carried out in the presence of Fab' fragments of the antibodies (Fig. 6). The Fab' fragment of the light chain-specific antibody (5F4) inhibited only the fluid phase activation and not the surface-mediated reaction. This suggests but does not prove the possibility that the mechanism of surface-mediated activation is different from that of fluid phase activation. In the fluid phase, Factor XIIa possibly interacts with Factor XI through its light chain region. In the surface-mediated reaction, in contrast, the interaction of Factor XIIa with Factor XI in the ternary complex involving HMW kininogen probably may occur through a different site on the Factor XI molecule. Further studies will be required to investigate these interesting possibilities. Finally, some consideration should be given to the manner by which the bivalent nature of Factor XI might influence the results of the studies reported here. It is unclear what function can be attributed to the unique dimeric structure of this coagulation protein. It has been demonstrated that each subunit of Factor XIa binds one molecule of HMW kininogen (30), which suggests that the stoichiometry of binding of HMW kininogen to Factor XI in plasma is 2:1. Although the stoichiometry of binding of our monoclonal antibodies to Factor XI is uncertain, we do not believe it is likely to influence the validity of the conclusions drawn from our present findings.

In conclusion, our studies, interpreted in the context of existing knowledge about the biochemistry of Factor XI, provide new insights concerning the relationship between the functional properties and the structural domains of Factor XI. Since the

light chain-specific antibody (5F4) inhibits both surface-mediated and fluid-phase proteolytic activation of Factor XI by Factor XIIa without inhibiting either the binding of Factor XI to HMW kininogen or the amidolytic activity of Factor XIa, we suggest the possibility (which requires further study) that (a) site(s) on the light chain of Factor XI may interact with Factor XIIa. Moreover, since the heavy chain-specific antibody (3C1) binds to an epitope near the HMW kininogen binding site, accelerates the rate of fluid-phase activation of Factor XI by Factor XIIa and substitutes for the presence of both surface and HMW kininogen, we suggest that this antibody may induce a conformational alteration in Factor XI that renders it a more favorable substrate for Factor XIIa. Finally, since this heavy chain-specific antibody also inhibits surface-mediated activation and binding of Factor XI to HMW kininogen, our studies strongly suggest that the activation of Factor XI by Factor XIIa in the presence of a surface is mediated by the binding to HMW kininogen of Factor XI through a site on the heavy chain region.

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References

1. Davie, E. W., K. Fujikawa, K. Kurachi, and W. Kisiel. 1979. The role of serine proteases in the blood coagulation cascade. *Adv. Enzymol.* 48:227-318.
2. Ratnoff, O. D. 1966. The biology and pathology of the initial coagulation reactions. *Prog. Hematol.* 5:204-245.
3. Iatridis, S. G., and J. H. Ferguson. 1962. Active Hageman factor. A plasma lysokinase of the human fibrinolytic system. *J. Clin. Invest.* 41:1277-1287.
4. Margolis, J. 1959. Activation of plasma by contact with glass. Evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol. (Lond.)* 144:1-22.
5. Griffin, J. H., and C. G. Cochrane. 1979. Recent advances in the understanding of contact activation reactions. *Semin. Thromb. Haemostasis.* 5:254-273.
6. Rapaport, S. I., R. R. Proctor, M. J. Patch, and M. Yeltra. 1961. The mode of inheritance of PTA deficiency: evidence for the existence of major PTA deficiency and minor PTA deficiency. *Blood.* 18:149-155.
7. Ragni, M. V., D. Sinha, F. Seaman, J. H. Lewis, J. A. Spero, and P. N. Walsh. 1985. Comparison of bleeding tendency, factor XI coagulant activity, and factor XI antigen in 25 factor XI-deficient kindreds. *Blood.* 65:719-724.
8. Bajaj, S. P. 1982. Cooperative Ca^{2+} binding to human factor IX. Effects of Ca^{2+} on the kinetic parameters of the activation of factor IX by factor XIa. *J. Biol. Chem.* 257:4127-4132.
9. Walsh, P. N., H. Bradford, D. Sinha, J. R. Piperno, and G. P. Tuszyński. 1984. Kinetics of the Factor XIa catalyzed activation of human blood coagulation Factor IX. *J. Clin. Invest.* 73:1392-1399.
10. Heeb, M. J., and C. P. Nova. 1985. Heavy chain of factor XIa is required for calcium-dependent cleavage of factor IX. *Thromb. Haemostasis.* 54:214(Abstr.)
11. Kurachi, K., and E. W. Davie. 1977. Activation of human factor XI (plasma thromboplastin antecedent) by factor XIIa (activated Hageman factor). *Biochemistry.* 16:5831-5839.
12. Bouma, B. N., and J. H. Griffin. 1977. Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *J. Biol. Chem.* 252:6432-6437.
13. Mannhalter, C., S. Schiffman, and A. Jacobs. 1980. Trypsin activation of human factor XI. *J. Biol. Chem.* 255:2667-2669.
14. Mandle, R. J., R. W. Colman, and A. P. Kaplan. 1976. Identification of prekallikrein and high molecular weight kininogen as a complex in human plasma. *Proc. Natl. Acad. Sci. USA.* 73:4179-4183.
15. Griffin, J. H., and C. G. Cochrane. 1976. Mechanisms for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor (Factor XII). *Proc. Natl. Acad. Sci. USA.* 73:2554-2558.
16. Thompson, R. E., R. Mandle, Jr., and A. P. Kaplan. 1977. Association of factor XI and high molecular weight kininogen in human plasma. *J. Clin. Invest.* 60:1376-1380.
17. Kurachi, K., K. Fujikawa, and E. W. Davie. 1980. Mechanism of activation of bovine factor XI by factor XII and factor XIIa. *Biochemistry.* 19:1330-1338.
18. Sinha, D., F. S. Seaman, A. Koshy, L. C. Knight, and P. N. Walsh. 1984. Blood coagulation Factor XIa binds specifically to a site on activated human platelets distinct from that for Factor XI. *J. Clin. Invest.* 73:1550-1556.
19. Kerbiriou, D. M., and J. H. Griffin. 1979. Human high molecular weight kininogen. *J. Biol. Chem.* 254:12020-12027.
20. Hojima, Y., J. V. Pierce, and J. J. Pisano. 1980. Hageman factor fragment inhibitor in corn seeds: purification and characterization. *Thromb. Res.* 20:149-162.
21. Kirby, E. P., and P. J. McDevitt. 1983. The binding of bovine factor XII to kaolin. *Blood.* 61:652-659.
22. March, S. C., I. Parikh, and P. Cuatrecasas. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149-152.
23. Sinha, D., A. Koshy, F. S. Seaman, and P. N. Walsh. 1985. Functional characterization of human blood coagulation factor XIa using hybridoma antibodies. *J. Biol. Chem.* 260:10714-10719.
24. Scott, C. F., D. Sinha, F. S. Seaman, P. N. Walsh, and R. W. Colman. 1984. Amidolytic assay of human factor XI in plasma: comparison with a coagulant assay and a new rapid radioimmunoassay. *Blood.* 63:42-50.
25. Proctor, R. R., and S. I. Rapaport. 1961. The partial thromboplastin time with kaolin. A simple screening test for first stage plasma clotting deficiencies. *Am. J. Clin. Pathol.* 36:212-219.
26. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)* 227:680-685.
28. Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849-857.
29. vanderGraaf, F., J. S. Greengard, B. N. Bouma, D. M. Kerbiriou, and J. H. Griffin. 1983. Isolation and functional characterization of the active light chain of activated human blood coagulation factor XI. *J. Biol. Chem.* 258:9669-9675.
30. Warn-Cramer, B. J., and S. P. Bajaj. 1985. Stoichiometry of binding of high molecular weight kininogen to factor XI/XIa. *Biochem. Biophys. Res. Commun.* 133:417-422.