Identification and Characterization of a Binding Site for Factor XIIa in the Apple 4 Domain of Coagulation Factor XI*

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Previously we have characterized a binding site for high M, kininogen in the first of four tandem-repeat (Apple) domains within the heavy chain region of factor XI (Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1990) J. Biol. Chem. 265, 4149–4154; Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1991) J. Biol. Chem. 267, 4247–4252), whereas a substrate binding site for factor IX was localized to the second Apple (A2) domain (Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1991) J. Biol. Chem. 266, 24190–24197). To define the factor XI domain that binds factor XIIa, we have screened a panel of synthetic peptides for their capacity to inhibit factor XI activation by factor XIIa. Peptide Gly³²⁵-Lys³⁵⁷ (located in the A4 domain) is a noncompetitive inhibitor of factor XI activation by factor XIIa (Kᵢ = 3.75 μM), whereas structurally similar peptides from the A1, A2, and A3 domains were required at >1000-fold higher concentrations for similar effects. The same peptide (Gly³²⁵-Lys³⁵⁷) is a competitive inhibitor of factor XIIa amidolytic activity (Kᵢ = 3.8 μM) suggesting that it binds near the active site of factor XIIa. Computer modeling was used to predict the secondary and tertiary structure of the A4 domain of factor XIIa (17). The present study was undertaken to determine whether domains in the heavy chain region of factor XI are important for activation by factor XIIa. Evidence is presented to support the conclusion that the A4 domain of factor XI is capable of interacting with factor XIIa.

Materials and Methods

Purification of Coagulation Proteins—Factor XI, purified from human plasma by immunoaffinity chromatography using a monoclonal antibody to factor XI (10), had a specific activity of 200 units/mg protein. Human α-factor XIIa was prepared and purified by the method of Shore et al. (19) and was a generous gift of Drs. Joseph D. Shore and M. Margarida Bernardo (Division of Biochemical Research, Henry Ford Hospital, Detroit, MI). The protein migrated at an apparent Mᵣ of 80,000 by SDS-polyacrylamide gel electrophoresis (PAGE) in the absence of reducing agents, whereas in the presence of reducing agents it migrated as a heavy chain of 50,000 Mᵣ and a light chain of 30,000 Mᵣ. The factor XIIa concentration in solution was estimated from its amidolytic activity. It hydrolyzed 3.78 × 10⁻⁴ mol of the chromogenic substrate S-2302 (S-Pro-Phe-Arg-p-nitroanilide, Kabi Diagnostica, Molndal, Sweden)/min/μg of protein at pH 8.0 and 37 °C. All purified proteins appeared homogeneous by SDS-PAGE. Human α-thrombin was a gift from Dr. John Fenton of the New York State Department of Health, Albany, NY. Activated protein C was donated by Drs. Mary Jo Heeb and John H. Griffin of the Scripps Research Institute, La Jolla, CA. Plasma kallikrein was purchased from Kabi Vitrum, Molndal, Sweden.

Peptide Synthesis—Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer by a modification of the procedure described by Kent and Clark-Lewis (20) as previously described (21). The sequences of the synthetic peptides utilized in this study are given in Table I.

Human plasma factor XI is a 160,000-dalton homodimer consisting of two identical disulfide-linked polypeptide chains each of which can be cleaved at a single peptide bond (Arg⁶⁰⁰-Val⁶⁰¹) by factor XIIa to give rise to factor XIa (1, 2). This

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Refolding and Reduction and Alkylation of Peptides—In order to refold peptides containing cysteine residues, the peptide was dissolved in deionized water as a 0.1 mg/ml solution in a flask containing a stir bar. The pH was adjusted to 8.5 with NH₄OH, and the solution was allowed to stir at 5 °C for at least 3 days. The resulting solution was lyophilized. Alternatively, peptides were reduced with dithiothreitol and alkylated with iodoacetamide as previously described (16).

High Performance Liquid Chromatography (HPLC)—The HPLC system employed was from Waters (Waters 600 Gradient Module, model 740 Data Module, model 46K Universal Injector, and Lambda-Max model 481 Detector). The reverse-phase chromatography described here was performed using a Waters C18 ,μBondapak Column, whereas gel filtration was carried out using a Waters Protein-Pak 60 Column as previously described (14, 17, 21).

Characterization of Synthetic Peptides—All the peptides utilized in this study were examined by HPLC (both reverse phase and gel filtration), and all demonstrated a single homogeneous peak (data not shown). When the refolded peptides were examined by HPLC (both reverse phase and gel filtration), single homogeneous peaks with identical retention times to the original mixtures were observed. This demonstrates the presence of a single homogeneous mixture of refolded peptides and not a mixed population of diverse polymers. The results were the same after reduction and alkylation of these same peptides with identical retention times to the original mixtures were observed by both reverse-phase and gel filtration HPLC. In addition, all reduced and alkylated or refolded peptides were examined for free –SH groups using the Ellman reagent, 5,5’-dithiobis(2-nitrobenzoic acid). It was determined (22) that there was less than 0.02 mol of free –SH/mole of peptide, which further verifies that all refolded peptides were homogeneous preparations consisting of intramolecular disulfide-bonded peptide.

Fast Atom Bombardment (FAB) Mass Spectrometry—FAB mass spectrometry was utilized to assess homogeneity and molecular mass of each synthesized peptide. FAB was performed as previously described (17, 21). The spectra of synthesized peptides revealed masses almost identical to calculated values (i.e. within 0.1%) with a single ion species detected in each case (data not shown).

Computer Modeling—A structural model of the A4 domain was constructed using the computational chemistry package supplied by Molecular Simulations, Inc., Pasadena, CA and a Silicon Graphics 4D/280 Parallel Processing Supercomputer. A description of the modeling package and methods has been previously published (21, 23). Information concerning cysteine disulfide constraints (24) was used to initiate model building after which extended energy minimization calculations were carried out according to previously published methods (14, 17, 21). Subdomains were selected for synthesis directly from models. Conformational constraints were built into selected peptides using artificially introduced cysteine pairs.

Coagulation Assays—Factor XI(a) was assayed by minor modifications (25) of the kaolin-activated partial thromboplastin time (26) using factor XI congenitally deficient substrate plasma, results of which were quantitated on double logarithmic plots of clotting times versus factor concentrations (13). Peptide effects in intravenous administration were measured using normal plasma.

Preparation and Characterization of Factor XIa—Purified factor XIa was activated by incubation at 37 °C with human factor XIIa as previously described (10) to use as a reference standard in assays of factor XIa generation. Gel electrophoresis in the presence of Na-DodSO₄ of this preparation under reducing conditions showed two major bands of Mr 50,000 and 30,000.

Amidolytic Assays—Factor XIa was assayed using the chromogenic substrate S-2302 according to the procedure previously described (27). The amidolytic assay of factor XIa was carried out by a modification of the method of Scott et al. (25). Factor XIa generation in the experiments reported here was quantitated from a standard curve prepared using purified factor XIa. Thrombin, trypsin (obtained from Sigma), and activated protein C at a concentration of 2 nM were assayed using 1 mM of the chromogenic substrate S-2302 (H-D-Phe-pip-Arg-pNA, Kabi). Plasma kallikrein (2 nM) was assayed using 1 mM of the chromogenic substrate S-2302 (H-D-Pro-Phe-Arg pNA, Kabi).

Assay of Factor XI Activation—The rate of factor XI activation by factor XIIa was determined as previously reported (11). Human factor XI (0.17 μM) was incubated with factor XIIa (0.017 μM) for 15 min at 37 °C, and the rate of factor XIa generation was measured using the substrate S-2302 at 405 nm.

Effect of Peptides on the Rate of Activation of Factor XI by Factor XIIa—The assay procedure was the same as described above except that factor XIa was incubated for 3 min at 37 °C with either peptide or buffer solution before the addition of other components of the assay mixture.

Kinetics of Activation of Factor XI by Factor XIIa—The assay was the same as described above except that the initial rates of activation were determined over a wide range of substrate concentrations. For determination of the effect of peptides on the kinetics of the reaction, the mixture was incubated with various concentrations of peptide for 3 min at 37 °C before the other components of the assay were added. The initial rates of release of factor XI activation were linear and were determined under conditions where less than 20% of the substrate had been consumed. Values for the Michaelis constant (Km) and the maximum velocity (Vmax) were obtained by the Lineweaver-Burk method (28) and were calculated using least-squares fit as previously described (16).

Analysis of Kinetic Data for the Quantitation of the Inhibitor Constants—The I50 method of Cha (29) was used to determine the inhibitor constants as previously described (16). In the case of classical competitive inhibition, I50 (total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) is related to the substrate concentration as follows: I50 = 1/2 E, + K, + KS/Km where E, equals the total enzyme concentration and S equals the substrate concentration. Thus, from the plot of I50 versus S, K (the inhibitor constant) can be determined. In the case of noncompetitive inhibition, I50 is independent of the substrate concentration and related to K as follows: I50 = 1/2 E, + K, + K, can be evaluated by determining I50 at several enzyme concentrations.

RESULTS

Effects of Heavy Chain-derived Peptides on the Activation of Factor XI by Factor XIIa—Our previous studies of the interactions of factor XI (XIa) with high M, kinogen (13, 14) and factor IX (17) have delineated binding sites for these two proteins within the carboxyl-terminal half of the first two Apple domains of factor XI. Preliminary computer modeling studies with all four Apple domains suggested that a similar pattern of folding might be present in each domain, A1–A4, each characterized by three stem-loop structures that might interact with one another to form a solvent-accessible contact surface (17). Such a surface, comprising residues Val35-Lys83, is apparently the high M, kinogen-binding site in the A1 domain of factor XI (10, 11), whereas a similar surface, comprising residues Ala124-Leu172, forms a substrate (factor IX) binding site in the A2 domain of factor XIa (12). Because of the structural similarities predicted from computer modeling and the 23–34% sequence identity among these four tandem repeat domains (2), we prepared a panel of heavy chain-derived peptides similar to those representing factor IX and high M, kinogen-binding sites (Table 1) to determine their effects on factor XI activation by factor XIIa. Peptide Gly325-Lys357 from the A4 domain inhibited factor XI activation by factor XIa 50% at a concentration of 5 μM (Fig. 1). In contrast, the concentration of the peptide derived from the A1 domain (Phe46-Ser98) required to produce 50% inhibition of factor XI activation was 7.5 mM (>1,000-fold higher concentration). Moreover, peptides from the A2 domain (Asn145, Ala186) and the A3 (Asn285, Arg292) were not effective even at a 10,000-fold higher concentration.

Effect of A4 Peptide (Gly325-Lys357) on Factor XIa Coagulant Activity—In order to determine if the A4-derived peptide Gly325-Lys357 inhibits factor XI coagulant activity, coagulant assays were carried out (see "Materials and Methods") in the presence of various concentrations of peptide. The concentration of Gly325-Lys357 required to inhibit factor XIa coagulant activity 50% was 7.5 × 10^-5 M, i.e. almost the same as the concentration required for 50% inhibition of factor XIa activation by factor XIa (data not shown).

Kinetics of Factor XI Activation by Factor XIIa in the Presence of A4 Peptide Gly325-Lys357—The Lineweaver-Burk plots of the activation of factor XIa by factor XIa in the presence of various concentrations of the heavy chain synthetic peptide Gly325-Lys357 are shown in Fig. 2A. The double-
The activity of Factor XIIa on the activation of factor XI by factor XIIa was examined using various synthetic peptides. Incubation of Factor XIIa with designated peptides for 3 min at 37 °C indicates that the catalytic site is blocked, thereby reducing the effective enzyme concentration.

To determine whether the A4 peptide (Gly^{326}-Lys^{357}) inhibits Factor XIIa, it was incubated with trypsin, kallikrein, α-thrombin, and activated protein C. Chromogenic assays demonstrated no significant inhibition of α-thrombin, trypsin, kallikrein, or activated protein C. The peptide Gly^{326}-Lys^{357} (data not shown) provided evidence of specificity for the A4 peptide as an inhibitor of Factor XIIa.

The inhibitory constant (K_i) for the A4 peptide (Gly^{326}-Lys^{357}) as an inhibitor of Factor XIIa was determined from double-reciprocal plots as described by Cha (29). The calculated value of K_i from the equation under "Materials and Methods." The inset of Fig. 2A shows that the I_50, which is independent of substrate concentration, is 3.75 μM. In the case of competitive inhibition, I_50 is related to the substrate concentration according to the equation under "Materials and Methods." It can be seen from this equation that a plot of I_50 versus substrate concentration should be a straight line, from which K_i can be determined.

**Factor XIIa Activation by Factor XIIa**

**TABLE 1**

| Tandem repeat residue number | Factor XI domain | Residue no. in Factor XI |
|-----------------------------|-----------------|-------------------------|
| 10                          | A1              | Phε^{266}-Ser^{296} (C)* |
| 50                          | A2              | Asn^{235}-Gly^{307} (C)* |
| 60                          | A3              | Gly^{326}-Arg^{356} (C)* |
| 70                          | A4              | Gly^{326}-Lys^{357} (C)* |
|                             | A5              | Asn^{235}-Lys^{357} (C)* |
| 80                          | Gly^{326}-Lys^{357} (C)* |

**Designates a residue where cysteine was substituted for an amino acid in the native factor XI sequence.**

**Designates a peptide in which cysteine replaced one or more amino acid(s) in the normal factor XI sequence.**

**Designates a residue where alanine was substituted for a cysteine in the native factor XI sequence.**

**Designates a residue where cysteine was substituted for one or more amino acid(s) in the normal factor XI sequence.**

**Designates a residue where alanine was substituted for a cysteine in the native factor XI sequence.**

**FIG. 1. Effects of factor XI heavy chain synthetic peptides on the activation of factor XI by factor XIIa.** The effects of various synthetic peptides on the activation of factor XI by factor XIIa were examined using Gly^{326}-Lys^{357} (A), Phε^{266}-Ser^{296} (B), Asn^{235}-Gly^{307} (C), and Gly^{326}-Arg^{356} (D). Factor XIIa (0.017 μM) was incubated for 3 min at 37 °C with the designated peptide at the concentration shown or with buffer solution. Then human factor XI (0.17 μM) was added, the mixture was incubated at 37 °C for 15 min, and the rate of factor XIa formation was determined as described under "Materials and Methods."

**The Effect of A4 Peptide on the Kinetics of the Amidolytic Activity of Factor XIIa Using the Substrate S-2302**—Because the catalytic site of factor XIIa is apparently blocked by the peptide Gly^{326}-Lys^{357}, we asked whether the amidolytic activity might also be affected. The Lineweaver-Burk plots of the amidolytic activity of factor XIIa using the substrate S-2302 in the presence of various concentrations of heavy chain peptide Gly^{326}-Lys^{357} are shown in Fig. 2B. The double-reciprocal plots yielded patterns consistent with classical competitive inhibition; that is, the V_max was unaffected by the peptide while progressively higher concentrations of peptide yielded commensurately increased slopes (i.e. higher values of K_m). Thus, the binding of the peptide to factor XIIa reduces the effective concentration of binding sites within the enzyme for its substrate S-2302.

In order to determine whether the A4 peptide (Gly^{326}-Lys^{357}) has inhibitory activity against serine proteases other than factor XIIa, the peptide was incubated with trypsin, kallikrein, α-thrombin, and activated protein C. Chromogenic assays of enzymatic activity demonstrated no significant inhibition of α-thrombin, trypsin, kallikrein, or activated protein C using up to 1 mM A4 peptide (Gly^{326}-Lys^{357}) (data not shown). Thus, this provides evidence of high degree of specificity of the A4 peptide (Gly^{326}-Lys^{357}) as an inhibitor of Factor XIIa.

**Calculation of Binding Constants from Kinetic Parameters**—The inhibitor constant K_i (the dissociation constant of the enzyme-inhibitor complex) was determined from the double-reciprocal plots as described by Cha (29). In the case of noncompetitive inhibition, I_50 is independent of substrate concentration and related to enzyme concentration according to the equation under "Materials and Methods." The inset of Fig. 2A shows that the I_50, which is independent of substrate concentration, is 3.75 μM. In the case of competitive inhibition, I_50 is related to the substrate concentration according to the equation under "Materials and Methods." It can be seen from this equation that a plot of I_50 versus substrate concentration should be a straight line, from which K_i can be determined. The inset of Fig. 2B represents such a plot with the factor XI heavy chain peptide Gly^{326}-Lys^{357} used as the inhibitor. The calculated value of K_i from the slope is 3.8 μM.

**Computer Modeling of the A4 Peptide Gly^{326}-Lys^{357}**—In order to gain more information about the three-dimensional structure of the Factor XIa-binding site on factor XI, we used computer modeling of the A4 domain to generate a testable three-dimensional structure (Fig. 3), using the primary amino acid sequence and the disulfide linkages (24) within the A4 domain (Phε^{271}-Lys^{357}). This model may or may not accurately reflect the solution structure of the factor XI A4 domain. However, it does predict a possible contact surface for interaction with factor XIIa. It is important to emphasize that the model is not presented to depict the structure of the A4 domain of factor XI, but rather as a hypothetical paradigm to be used to design functionally active synthetic peptides. The calculated structure shows three antiparallel β-strands connected by β-turns defined by amino acid residues Ala^{235}-Gly^{307}, Lys^{351}, Lys^{359}, and Gly^{344}, Gly^{357}.

**Effects of Stem-loop Synthetic Peptides on the Activation of Factor XI by Factor XIIa**—In order to determine whether the three stem-loops assume a conformation that comprises a contact site that interacts with factor XIIa, we prepared synthetic peptides, rationally designed on the basis of the model (Fig. 3). In some of the peptides, cysteines were introduced so that the resulting disulfide bond might stabilize this loop structure in a conformation likely to resemble the native
binding surface. These peptides were examined for their capacity to inhibit the activation of factor XI by factor XIIa after refolding or reduction and alkylation (see "Materials and Methods"). The peptide designated Ala$^{17}$-Gly$^{26}$ in which cysteines were substituted, inhibited 50% the activation of factor XI by factor XIIa at a concentration of $8 \times 10^{-8}$ M when subjected to a folding procedure (see "Materials and Methods") and lost significant inhibitory activity when reduced and alkylated (Fig. 4 and Table II), suggesting that the correct conformation of this peptide is required for optimal binding. This supports the suggestion that this first loop constitutes a part of the surface of the factor XIIa-binding site.

Since synthetic peptide Gly$^{326}$-Lys$^{350}$ inhibits the activation of factor XI by factor XIIa (Fig. 1) and since the computer model (Fig. 3) suggests the possibility that the stem-loop structures Lys$^{331}$-Lys$^{340}$ and Gly$^{341}$-Gly$^{350}$ form a portion of the factor XIIa-binding site, we synthesized conformationally unconstrained Lys$^{331}$-Lys$^{340}$ and a conformationally constrained Gly$^{341}$-Gly$^{350}$ (Table I). The folded conformation of Gly$^{341}$-Gly$^{350}$ (C) was more than 100-fold more effective as an inhibitor of factor XI activation by factor XIIa than the reduced and alkylated peptide (Fig. 4 and Table II). However, the unconstrained peptide Lys$^{331}$-Lys$^{340}$ representing the second stem loop did not inhibit the activation of factor XI by factor XIIa when used at concentrations up to $10^{-4}$ M. Because our model (Fig. 3) predicted that the distance between $\alpha$-carbons of amino acids within the antiparallel $\beta$-strands (stem portion of stem-loop 2) was greater than 10 Å, we were reluctant to constrain this region by introduction of cysteines, since the distance between $\alpha$-carbons of disulfide-bonded cysteines is approximately 6 Å. Thus, the failure of the constrained Lys$^{331}$-Lys$^{340}$ peptide to inhibit factor XIIa-mediated factor XI activation could be attributed to the failure of the peptide to assume the correct conformation. This possibility was borne out by additional experiments described below. Another peptide representing a sequence of amino acids from the amino-terminal half of the A4 domain (Asp$^{278}$, Gly$^{296}$) (C), not implicated by the computer model to contribute to the putative binding surface, failed to affect factor XIIa-catalyzed factor XI activation at concentrations as high as 1 mM (Table II).

Effects of Peptide Combinations on the Activation of Factor XI by Factor XIIa—The results presented in Fig. 4 together with the model (Fig. 3) suggest that Ala$^{17}$-Gly$^{26}$ and Gly$^{44}$-Gly$^{50}$ form stem-loop structures that contribute to the formation of a binding site for factor XIa. It was, therefore, important to examine the inhibitory effects of these peptides when added in combination at equimolar concentrations. Fig. 4B indicates that using equimolar mixtures of folded Ala$^{17}$-Gly$^{26}$ (C) and Gly$^{44}$-Gly$^{50}$ (C) the inhibitory effects observed were strictly additive, compared with the effects of either peptide examined alone. Similarly when all three peptides, i.e. folded Ala$^{17}$-Gly$^{26}$ (C) plus folded Gly$^{44}$-Gly$^{50}$ (C) plus Lys$^{331}$-Lys$^{340}$, were added at equimolar concentrations the inhibitory effects were similar to those obtained with the longer peptide encompassing two of the putative stem loops (Gly$^{326}$-Lys$^{350}$). Therefore, an additional experiment was performed to determine whether Lys$^{331}$-Lys$^{340}$ is active in inhibiting the activation of factor XI by factor XIIa when folded Ala$^{17}$-Gly$^{26}$ (C) and Gly$^{44}$-Gly$^{50}$ (C) are present. Fig. 5 shows that when the concentration of Lys$^{331}$-Lys$^{340}$ is increased from $10^{-8}$ M to $10^{-4}$ M while the combined concentration of folded Ala$^{17}$-Gly$^{26}$ (C) and folded Gly$^{44}$-Gly$^{50}$ (C) is increased from 0 to $10^{-5}$ M, evidence of inhibitory effects of Lys$^{331}$-Lys$^{340}$ is disclosed. Thus, it is apparent that concentrations of $10^{-1}$ M to $10^{-5}$ M Lys$^{331}$-Lys$^{340}$, which by themselves have no inhibitory effects, enhance the inhibitory effects of Ala$^{17}$-Gly$^{26}$ (C) plus Gly$^{44}$-Gly$^{50}$ (C), e.g. 10% inhibition at $10^{-7}$ M combined peptides becomes 60% inhibition and 50% inhibition at $10^{-8}$ M combined peptides becomes 100% in the presence of Lys$^{331}$-Lys$^{340}$ which by itself has no inhibitory effect. Therefore, it would appear that the three peptides interact in a complex way either with each other or with factor XIIa or both to potentiate the inhibition of factor XIIa-catalyzed factor XI activation. As a final test of this hypoth-
FIG. 3. Computer model of the A4 domain of the heavy chain of factor XI. A plausible three-dimensional structure was calculated using the primary structure of the A4 domain, known disulfide linkages, Biograf software (Molecular Simulations, Inc.), and a Silicon Graphics 4D 280 Parallel Processing Computer (see "Materials and Methods"). A stereographic pair of α-carbon tracings of the protein backbone is shown with the positions of amino acids numbered according to their sequence in the mature protein.

The participation of factor XI in the contact phase of blood coagulation involves intermolecular interactions with factor XIIa, high Mγ, kininogen, and factor IX. Our most recent experiments have resulted in the identification and characterization of conformationally specific solvent-accessible contact surfaces within the first two Apple domains of the factor XI heavy chain that form binding sites for high Mγ, kininogen, and factor IX (13, 14, 17). Previous studies in our laboratory using monoclonal antibodies have revealed that domains in both the heavy chain and light chain are essential for the efficient activation of factor XI by factor XIIa (13). In order to gain information about the mechanisms of activation of factor XI by factor XIIa and to identify and characterize the domains and amino acid sequences within factor XI that interact with factor XIIa, the present study was undertaken.

The results of our experiments support the hypothesis that a sequence of amino acids (Ala<sup>317</sup>-Gly<sup>326</sup>) in the A4 domain of the heavy chain may contain three antiparallel β-strands connected by β-turns, which comprise a continuous surface that interacts with factor XIIa. The evidence supporting this possibility is listed as follows: 1) an A4-derived peptide (Gly<sup>296</sup>-Lys<sup>307</sup>) inhibits factor XI activation by factor XIIa noncompetitively with a K<sub>i</sub> of 3.75 μM (Figs. 1 and 2). This same peptide is a competitive inhibitor (K<sub>i</sub> = 3.8 μM) of factor XIIa amidolytic activity (Fig. 2) suggesting that it binds near the active site of factor XIIa. 2) A model of the A4 domain derived from energy minimization computations (Fig. 3) predicts the presence of β-stranded loop structures (Ala<sup>317</sup>-Gly<sup>326</sup>, Lys<sup>311</sup>-Lys<sup>314</sup>, and Gly<sup>344</sup>-Gly<sup>352</sup>) that fold together to form a solvent-accessible surface that might comprise a binding site for factor XIIa. 3) Based on this model, two conformationally constrained peptides and one peptide without conformational constraints were synthesized. Both of the constrained peptides (Ala<sup>317</sup>-Gly<sup>326</sup> and Gly<sup>344</sup>-Gly<sup>352</sup>) inhibited the activation of factor XI by factor XIIa when refolded and lost inhibitory activity when reduced and alkylated (Fig. 4A). 4) The unconstrained peptide Lys<sup>311</sup>-Lys<sup>314</sup> (inactive by itself) acted to potentiate the inhibitory effects of the constrained peptides (Ala<sup>317</sup>-Gly<sup>326</sup> and Gly<sup>344</sup>-Gly<sup>352</sup>) on the activation of factor XI by factor XIIa whereas when the two constrained peptides were added at equimolar concentrations simple additive inhibitory effects were demonstrated (Figs. 4B and 5). 5) A conformationally constricted peptide (Ala<sup>317</sup>-Gly<sup>326</sup>) containing all three putative β-stranded loop structures was the most effective inhibitor of factor XI activation by factor XIIa (Fig. 4B and Table II). These experiments demonstrate that the optimal function of the factor XIIa site requires the presence and the proper conformation of all three putative β-stranded loop structures.

In our approach to this problem, in the absence of any crystallographic or solution structural information about the Apple domains of factor XI, we have used computer modeling to predict the secondary and tertiary structure of the A4 domain of factor XI that appears to form a contact surface with factor XIIa. The molecular dynamic, energy minimization calculations suggested that this domain may have a
structural motif consisting of three antiparallel $\beta$-strands connected by $\beta$-turns. This construct was used to design peptides restricted with disulfide bonds, and we have shown that their activity (i.e., their ability to inhibit factor XI activation by factor XIIa) is dependent on their conformation. Thus, the model has been used as a tool to generate hypotheses, about the possible secondary and tertiary structure of the A4 domain, that were tested in functional studies of factor XI activation. Although our results are consistent with the possibility that the predicted structural motif has some validity, the model is not presented as evidence of the three-dimensional structure of the A4 domain, which can be obtained only using physical techniques such as x-ray crystallography or nuclear magnetic resonance. However, it will be interesting to determine whether the model makes correct structural predictions.

We have investigated refolded peptides and reduced and alkylated preparations and have found they have identical retention times (by gel filtration and reverse-phase HPLC) thus suggesting they are homogeneous species and that polymerization does not account for their inhibitory effects on factor XI activation. Also, all refolded peptides and all reduced and alkylated peptides contained no free thiols, thus, free $\beta$-SH groups were confirmed to be either oxidized to disulfides or reduced and alkylated. Thus, from the refolding experiments we conclude that protein conformation is an important determinant of the structure of the surface that binds factor XIIa.

We have carried out similar experiments that identified binding sites for high $M$, kininogen (13, 14) within domain A1 and for factor IX (17) in domain A2. The binding site for factor IX in the A2 domain of the heavy chain also contains three putative antiparallel $\beta$-strands connected by $\beta$-turns which comprise a continuous surface. The A2 peptide comprising the sequence of amino acids, Ala$^{124}$-Leu$^{172}$, was found to be a competitive inhibitor of factor IX activation by factor XIa and was therefore inferred to represent a substrate-binding site for factor IX. In contrast, the A4 peptide Gly$^{396}$-Lys$^{346}$ is a noncompetitive inhibitor of factor XI activation by factor XIIa (Fig. 2A). Furthermore, this same peptide is a competitive inhibitor of factor XIa amidolytic activity suggesting that it binds near the active site of factor XIIa (Fig. 2B). The demonstration that the A4 peptide Gly$^{396}$-Lys$^{346}$ has no inhibitory effects on the amidolytic activities of a variety of serine proteases other than factor XIa (including thrombin, activated protein C, trypsin, and kallikrein) indicates that it binds specifically to factor XIIa and has nonspecific inhibitory effects. Thus, it is clear from our experiments that the A4 domain of factor XI contains amino acid sequences Ala$^{124}$-Gly$^{172}$ that are important in binding factor XIa and play a role in the activation of factor XI by factor XIIa.
A recent report by Meijers et al. (30) provides definitive evidence that the A4 domain of factor XI mediates dimer formation. Previously it was known that factor XI is present in plasma as a homodimer (1, 2) and is secreted as a dimer linked by a disulfide bridge between Cys321 of each A4 domain (24). Interestingly, whereas recombinant factor XI-Cys321 Ser migrated as a monomer by SDS-PAGE, gel filtration studies indicated that the protein exists as a dimer under native conditions (30). Furthermore, when the A4 domain was inserted into tissue plasminogen activator, dimer formation under native conditions was also observed, whereas neither the A3 domain of factor XI nor the A4 domain of prekallikrein promoted dimer formation. Our present studies indicate that in addition to mediating dimer formation the A4 domain also participates in factor XI activation by factor Xlla.

The mechanisms by which the A4 domain peptides inhibit the enzymatic activities of factor Xlla are somewhat conjectural. However, several inferences concerning the mechanisms of factor Xlla-catalyzed factor XI activation can be drawn from our studies within the context of the general theory of enzyme activation and kinetics. Enzyme-catalyzed reactions are characterized by the phenomenon of saturation of reaction rates as a function of substrate concentration, a feature not generally observed in nonenzymatic reactions. From this saturation effect it has been deduced that the enzyme first forms a reversible complex with the substrate through a substrate-binding site on the enzyme. Only when the substrate is prekallikrein promoted dimer formation. Our present studies indicate that a small peptide substrate-binding exosite presumably near the active site of factor Xlla, apparently recognized by the chromogenic substrate S-2302 and by the factor XI-derived A4 peptides. The answer to this question is somewhat conjectural, but it seems reasonable to suggest that it represents a secondary substrate-binding site that serves to position the factor XI cleavage site (Arg406-Ile470) near the active site of factor Xlla once the two proteins are already docked through binding of factor XI to a macromolecular substrate binding site in factor Xlla.

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