

# A Binding Site for Thrombin in the Apple 1 Domain of Factor XI\*

(Received for publication, July 10, 1995, and in revised form, November 22, 1995)

Frank A. Baglia‡ and Peter N. Walsh‡§¶

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

Previously we defined a binding site for high molecular weight kininogen (HK) in the A1 domain of factor XI (FXI). Since thrombin can activate FXI and HK inhibits the activation of FXI by thrombin, we have identified a thrombin binding site in FXI. Both the recombinant A1 domain (Glu<sup>1</sup>–Ser<sup>90</sup>) and a synthetic peptide (Phe<sup>56</sup>–Ser<sup>86</sup>) containing the HK binding site inhibited FXI activation by thrombin. Both a monoclonal antibody, 5F7, recognizing the A1 domain, and the rA1 domain were shown to be competitive inhibitors of thrombin-catalyzed FXI activation. The peptides Ala<sup>45</sup>–Arg<sup>54</sup> and Val<sup>59</sup>–Arg<sup>70</sup> acted synergistically to inhibit FXI activation by thrombin. Mutant rA1 domain constructs (Val<sup>64</sup> → Ala and Ile<sup>77</sup> → Ala), which do not inhibit FXI binding to HK, retain full capacity to inhibit FXI activation by thrombin. The peptide Ala<sup>45</sup>–Arg<sup>54</sup> inhibited thrombin-catalyzed FXI activation, whereas it had no effect on FXI binding to HK. In contrast, the peptide Asn<sup>72</sup>–Leu<sup>83</sup> (which inhibited FXI binding to HK) did not inhibit FXI activation by thrombin. Thus, a thrombin binding site exists in the A1 domain of FXI spanning residues Ala<sup>45</sup>–Arg<sup>70</sup> that is contiguous with but separate and distinct from the HK binding site. These sites may regulate which ligand is bound to FXI and through which pathway FXI is activated.

Factor XI (FXI)<sup>1</sup> is a homodimeric plasma glycoprotein that circulates as a complex with its cofactor high molecular weight kininogen (HK) (1, 2) and is proteolytically activated on negatively charged surfaces by FXIIa to give rise to FXIa (3–10). The mechanism, involving interactions of FXII, prekallikrein (PK), and HK, by which contact activation is initiated and its significance *in vivo* have yet to be established, since individuals congenitally deficient in any one of these contact factors (FXII, HK, and PK) do not experience abnormal bleeding, suggesting that these proteins are not required for coagulation *in vivo* (11, 12). In contrast, a deficiency of FXI can result in excessive bleeding after trauma or surgery (13, 14). These observations suggest that FXI may be activated *in vivo* by a protease(s) other than FXIIa.

\* This study was supported by research grants from the National Institutes of Health (HL46213, HL45486, and HL25661) and from the W. W. Smith Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 North Broad St., Philadelphia, PA 19140. Tel.: 215-707-4375; Fax: 215-707-3005.

<sup>1</sup> The abbreviations used are: FXI, FXIIa, FXIa, FXII, and FIX, factors XI, XIIa, XIa, XII, and IX, respectively; A1, Apple 1; rA1, recombinant Apple 1; HK, high molecular weight kininogen; PK, prekallikrein; HPLC, high performance liquid chromatography; pNA, *p*-nitroanilide;  $V_{max}$ , maximum velocity;  $K_i$ , inhibition constant;  $K_d$ , dissociation constant.

The ability of thrombin, an enzyme generated late in the coagulation cascade, to activate FXI has been demonstrated (15, 16). The site at which FXI is cleaved by thrombin is identical to that cleaved by FXIIa (16, 17). Determination of the kinetic parameters of FXI activation by thrombin and FXIIa indicate that at a physiological concentration of FXI, in the presence of dextran sulfate, thrombin would be the more potent activator (16). Although FXI is readily activated by thrombin in a purified system with dextran sulfate present, the reaction may not proceed as readily in plasma (15, 16, 18), since although HK promotes the FXIIa-mediated reaction it inhibits thrombin-catalyzed activation of FXI (15, 16, 18). These observations raise the following two related questions. Is thrombin a physiological activator of FXI in plasma? What is the mechanism by which HK can inhibit thrombin-catalyzed FXI activation?

The present study was undertaken to determine the sequence of amino acids in FXI that mediate its interaction with thrombin. Clarification of the mechanism of interaction of these two proteins might also help to elucidate the physiological importance of thrombin-catalyzed FXI activation. Four tandem repeat sequences (designated A1, A2, A3, and A4 or Apple domains) are present in the heavy chain of FXI (7). We have previously reported evidence for the presence of an HK binding site in the A1 domain (19, 20), a binding site for FXIIa in the A4 domain (21), a substrate binding site for FIX in the A2 domain (22), and recently, a specific binding site for platelets in the A3 domain (23). Evidence for a binding site in the A1 domain of FXI that is important for interaction with thrombin is reported in the present study.

## MATERIALS AND METHODS

**Purification of Proteins**—FXI (250 units/mg of protein) was purified from human plasma by immunoaffinity chromatography (24). The recombinant A1 (rA1) domain (Glu<sup>1</sup>–Ser<sup>90</sup>) and the rA2 domain (Ser<sup>90</sup>–Ala<sup>181</sup>) were prepared in *Escherichia coli* using the QIAexpress pQE-9 expression vector (Qiagen Inc., Chatsworth, CA). PK was purified as described (25). FXI was assayed by minor modifications (26) of the kaolin-activated partial thromboplastin time (27). Human  $\alpha$ -thrombin (2,800 NIH units/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). All purified proteins appeared homogeneous by SDS-polyacrylamide gel electrophoresis.

**Peptide Synthesis**—Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer by a modification (29) of the procedure described by Kent and Clark-Lewis (28). The sequences of the synthetic peptides utilized in this study are given in Table I. All the peptides utilized in this work were rationally designed, conformationally constrained synthetic peptides based upon a previously published (20) molecular model for the A1 domain of FXI. Each peptide was separately modeled using energy minimization calculations (20) that confirmed that the modeled peptides assumed a conformation similar to that of the A1 domain.

**Refolding and Reduction and Alkylation of Peptides**—A previously published method (24) was used to refold peptides containing cysteine residues. Alternatively, peptides were reduced with dithiothreitol and alkylated with iodoacetamide as described previously (24).

**High Performance Liquid Chromatography (HPLC)**—The HPLC system employed was from Waters (Waters 600 Gradient Module, model

TABLE I  
Synthetic peptides derived from the known sequence of factor XI or prekallikrein and the tandem repeats of which they are a part

		Tandem repeat residue number <sup>a</sup>										PK or FXI domain		Designation of peptide
45	50	60	70	80										
A E S P S	E D P T R	W F T C V L K D S V T E T L P R V	— N R T A A I S G Y S F K Q C S									FXI A1		Ala <sup>45</sup> —Ser <sup>86</sup>
A E S P C <sup>b</sup>	E D P C <sup>b</sup> R											FXI A1		Ala <sup>45</sup> —Arg <sup>54</sup> (C) <sup>c</sup>
		F T C V L K D S V T E T L P R V	— N R T A A I S G Y S F K Q C S									FXI A1		Phe <sup>56</sup> —Ser <sup>86</sup>
		V L K C <sup>b</sup> S V T E C <sup>b</sup> L P R										FXI A1		Val <sup>59</sup> —Arg <sup>70</sup> (C) <sup>c</sup>
		V L K D S V A <sup>d</sup> E T L P R										FXI A1		Val <sup>59</sup> —Arg <sup>70c</sup>
A E S P S	E D P T R											FXI A1		Ala <sup>45</sup> —Arg <sup>54</sup>
A E S P C <sup>b</sup>	A <sup>d</sup> D P C <sup>b</sup> R											FXI A1		Ala <sup>45</sup> —Arg <sup>54</sup> (C) <sup>c</sup>
A E S P C <sup>b</sup>	E A <sup>d</sup> P C <sup>b</sup> R											FXI A1		Ala <sup>45</sup> —Arg <sup>54</sup> (C) <sup>c</sup>
	C <sup>b</sup> E D P C <sup>b</sup>											FXI A1		Ser <sup>49</sup> —Thr <sup>53</sup> (C) <sup>c</sup>
												FXI A1		Asn <sup>72</sup> —Lys <sup>83</sup> (C) <sup>c</sup>
												FXI A2		Asn <sup>145</sup> —Ala <sup>176</sup>
												FXI A3		Asn <sup>235</sup> —Arg <sup>266</sup>
												FXI A4		Gly <sup>326</sup> —Lys <sup>357</sup>
												PK A1		Phe <sup>56</sup> —Gly <sup>86</sup>
P A S S I N D M E K												PK A1		Pro <sup>45</sup> —Lys <sup>54</sup>

<sup>a</sup> The numbers indicate the length of each peptide as reported by Fujikawa *et al.* (7). A gap was inserted in the A1 domain at residue 72 to maintain maximal alignment of residues.

<sup>b</sup> Designates a residue in which cysteine replaced for an amino acid in the normal factor XI sequence.

<sup>c</sup> Designates a peptide in which cysteine replaced one or more amino acid(s) in the normal factor XI sequence.

<sup>d</sup> Designates a peptide in which alanine replaced one or more amino acid(s) in the normal factor XI sequence.

740 Data Module, model 46K Universal Injector, and Lambda-Max model 481 Detector; Milford, MA). Reverse phase chromatography was performed using a Waters C8  $\mu$ Bondapak Column, whereas gel filtration was carried out using a Waters Protein-Pak 60 column as described previously (20, 22, 29).

**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, demonstrating the presence of a single homogeneous mixture of refolded peptides. The results were the same after reduction and alkylation of these same peptides. 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 (30) that there was less than 0.02 mol of free SH/mol of peptide, which further verifies that these refolded peptides were homogeneous preparations consisting of intramolecular disulfide-bonded peptide.

**Assay of Factor XI Activation**—Activation of FXI (60 nM) by thrombin (1.25 nM) was measured by chromogenic assay. Incubations were carried out in 200  $\mu$ l of 50 mM Tris, 150 mM NaCl, pH 7.3, with 1% bovine serum albumin (TBSA) and dextran sulfate (average molecular weight = 500,000) (Sigma) (1  $\mu$ g/ml) at 37 °C. After diluting to a final volume of 1 ml with TBSA containing 600  $\mu$ M S-2366 (Glu-Pro-Arg-pNA, Chromogenix, Mölndal, Sweden), the amount of free paranitroaniline was determined by measuring the change in absorbance at 405 nm (A405). The amount of FXIa generated was assayed by reference to a standard curve constructed using purified FXIa.

**Effect of Peptides on the Rate of Activation of Factor XI by Thrombin**—The assay procedure was the same as described above except thrombin (1.25 nM) was incubated for 5 min at 37 °C with either peptide or buffer solution before the addition of FXI (60 nM).

To determine whether the A1 domain peptides (Glu<sup>1</sup>—Ser<sup>90</sup>, Phe<sup>56</sup>—Ser<sup>86</sup>, Ala<sup>45</sup>—Arg<sup>54</sup>) inhibit the activity of  $\alpha$ -thrombin, the peptide was incubated with  $\alpha$ -thrombin for 5 min at 37 °C, and the mixture was assayed using the chromogenic substrate S-2238 (H-D-Phe-pip-Arg-pNA, Kabi Vitrum, Stockholm, Sweden) at concentrations of 3  $\mu$ M, 10  $\mu$ M, and 1 mM.

To determine whether the A1-derived peptides inhibit the enzymatic activity of thrombin in the conversion of fibrinogen to fibrin, an established procedure (31) was employed in which 0.1 ml of human fibrinogen (Sigma), 1.5 mg/ml was diluted in 0.8 ml of 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, to which was added 0.1 ml of  $\alpha$ -thrombin (5 units/ml) that had previously been incubated with A1 domain peptides Glu<sup>1</sup>—Ser<sup>90</sup>, Phe<sup>56</sup>—Ser<sup>86</sup>, or Ala<sup>45</sup>—Arg<sup>54</sup>. The increase in turbidity was recorded for 10 min.

**Kinetics of Activation of Factor XI by Thrombin**—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 effects of peptides or antibodies on the kinetics of the

reaction, the FXI was incubated with various concentrations of peptide or antibody for 10–15 min at 37 °C before the addition of thrombin. Values for the Michaelis constant ( $K_m$ ) and the maximum velocity ( $V_{max}$ ) were obtained by the Lineweaver-Burk method (32) and were calculated using least-square fit as described previously (33, 34).

**Analysis of Kinetic Data for the Quantitation of the Inhibitor Constants**—The  $I_{50}$  method of Cha (35) was used to determine the inhibitor constants as described previously (14). In the case of classical competitive inhibition,  $I_{50}$  (total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) is related to the substrate concentration as follows:  $I_{50} = \frac{1}{2} E_t + K_i + K_i S/K_m$ , where  $E_t$  equals the total enzyme concentration and  $S$  equals the substrate concentration. Thus, from the plot of  $I_{50}$  versus  $S$ ,  $K_i$  can be determined.

## RESULTS

**Effect of Monoclonal Antibodies on the Activation of Factor XI by Thrombin**—Previously, four monoclonal antibodies have been developed and characterized that have been useful in elucidating structure-function relationships of FXI (24, 33, 36). We initially determined the effects of binding of these antibodies to FXI on thrombin-catalyzed FXI activation. Fig. 1A shows that monoclonal antibody 5F7 can completely block thrombin-catalyzed FXI activation with 50% inhibition at  $\sim 5 \times 10^{-10}$  M 5F7, a concentration close to the  $K_d$  ( $6.2 \times 10^{-10}$  M) for 5F7 binding to FXI (36). Antibody 5F7 binds to the A1 domain of FXI (19) and blocks surface-mediated activation of FXI and its binding to HK (36). The specificities of the other four antibodies are indicated in the legend to Fig. 1. The concentration of 3C1 monoclonal antibody required to inhibit thrombin-catalyzed FXI binding was 100-fold greater ( $5 \times 10^{-8}$  M) than that of the 5F7 antibody (Fig. 1A), whereas antibodies 1F1 and 5F4 had no effect. This experiment suggests that a binding site for thrombin exists in the A1 domain of FXI.

**Effects of Heavy Chain Peptides on the Activation of Factor XI by Thrombin**—It is well documented that *in vitro* FXI (60 nM) can be proteolytically activated by thrombin (1.25 nM) to generate FXIa (15, 16). Since the 5F7 monoclonal antibody inhibited thrombin-catalyzed FXI activation (Fig. 1A), we determined whether peptides derived from amino acid sequences of the heavy chain of FXI could affect the activation of FXI by thrombin. The rA1 domain (Glu<sup>1</sup>—Ser<sup>90</sup>) inhibited the activation of FXI by thrombin with an  $IC_{50}$  of  $5 \times 10^{-6}$  M, whereas a conformationally constrained synthetic peptide (Phe<sup>56</sup>—Ser<sup>86</sup>) containing the HK binding site (19, 20) also inhibited thrombin-catalyzed FXI activation ( $IC_{50} = 10^{-4}$ ) (Fig. 1B). In contrast, neither the rA2 domain (Ser<sup>90</sup>—Ala<sup>181</sup>, containing a sub-

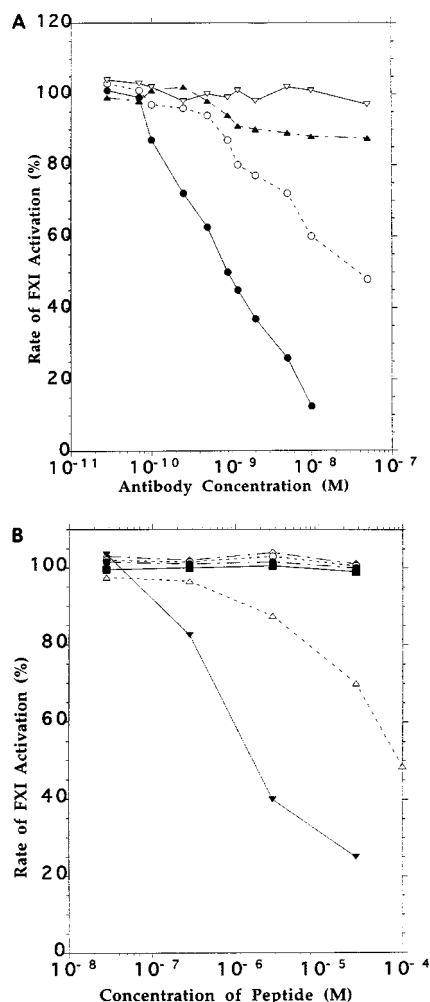


FIG. 1. Effects on the rate of activation of FXI (60 nM) by thrombin (1.25 nM) of the presence of various concentrations of monoclonal antibodies (A) or heavy chain-derived peptides (B). FXI was incubated with either buffer or various concentrations of the antibody solution or peptides prior to use in the assay. The rate of FXIa formation was determined as described under "Materials and Methods." A, data shown are those obtained with monoclonal antibodies, 5F7 (●—●), 3C1 (○—○), 1F1 (△—△), and 5F4 (▽—▽). Antibody 5F7 is immunologically reactive to the A1 domain of FXI (19). Antibody 3C1 is heavy chain-specific, is immunologically cross-reactive with the A2 domain (36), and is a competitive inhibitor of FXI activation by FXIa (24, 33). Antibody 5F4 is light chain-specific (24, 33). Antibody 1F1 is also directed against the heavy chain of FXI (24). B, data shown are those obtained with Gly<sup>326</sup>-Lys<sup>356</sup> (◇—◇), Ala<sup>134</sup>-Ala<sup>176</sup> (○—○), Asn<sup>235</sup>-Arg<sup>266</sup> (●—●), rA2, Ser<sup>90</sup>-Ala<sup>181</sup> (■—■), Phe<sup>56</sup>-Ser<sup>86</sup> (△—△), and rA1, Glu<sup>1</sup>-Ser<sup>90</sup> (▽—▽).

strate binding site for FIX; Ref. 22 and Fig. 2) nor synthetic peptides representing sequences in the A3 domain (Asn<sup>235</sup>-Arg<sup>266</sup>, a platelet receptor binding site; Ref. 23) and A4 domain (Gly<sup>326</sup>-Lys<sup>356</sup>, an FXIIa binding site; Ref. 21) were able to inhibit the activation of FXI by thrombin (Fig. 1B). The rA1 domain peptide and other A1-derived peptides (including Phe<sup>56</sup>-Ser<sup>86</sup> and Ala<sup>45</sup>-Arg<sup>54</sup>) were examined for their capacity to inhibit the amidolytic activity of thrombin and the thrombin-catalyzed conversion of fibrinogen to fibrin (see "Materials and Methods"). No thrombin-inhibitory effects were observed at peptide concentrations up to 1 mM (data not shown), indicating that the inhibitory effects of these peptides are specific for thrombin-catalyzed FXI activation. Thus, these experiments reveal that a binding site for thrombin exists in the A1 domain of FXI, possibly near the binding site for HK in the A1 domain (Phe<sup>56</sup>-Ser<sup>86</sup>).

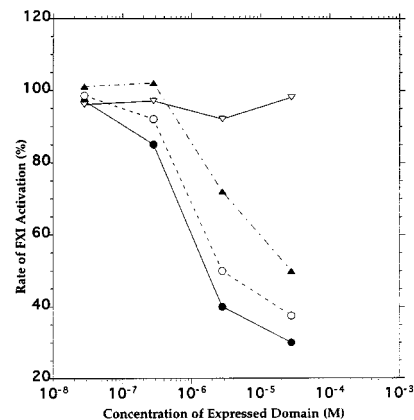


FIG. 2. Effects on the rate of activation of FXI (60 nM) by thrombin (1.25 nM) of the presence of various concentrations of recombinant peptides. Thrombin was incubated with either buffer or various concentrations of peptides prior to addition to the assay. The rate of FXIa formation was determined as described under "Materials and Methods." Data shown are those with Glu<sup>1</sup>-Ser<sup>90</sup> (●—●), Glu<sup>1</sup>-Ser<sup>90</sup> (Ile<sup>77</sup> → Ala<sup>77</sup>) (○—○), Glu<sup>1</sup>-Ser<sup>90</sup> (Val<sup>64</sup> → Ala<sup>64</sup>) (△—△) and Ser<sup>90</sup>-Ala<sup>181</sup> (▽—▽).

Previously we have identified specific amino acid residues within the A1 domain involved in binding HK (19, 20, 37). Utilizing mutational analysis we have determined that the binding of FXI to HK is mediated at least in part by Val<sup>64</sup> and Ile<sup>77</sup> in the A1 domain of FXI (37). Therefore, we examined the effects of mutations of these two residues on the capacity of the rA1 domain to inhibit thrombin-catalyzed FXI activation. We found that mutant rA1 domain constructs (Val<sup>64</sup> → Ala and Ile<sup>77</sup> → Ala), which have lost the capacity to inhibit FXI binding to HK (37), retain the full capacity of the rA1 domain (Glu<sup>1</sup>-Ser<sup>90</sup>) to inhibit thrombin-catalyzed FXI activation (Fig. 2). Therefore, the binding sites for HK and thrombin in the A1 domain, although contiguous, are apparently separate and distinct. Another experiment that supports this conclusion is that after reduction and alkylation, the rA1 domain (Glu<sup>1</sup>-Ser<sup>90</sup>) retains the capacity to inhibit FXI binding to HK ( $IC_{50} \sim 10^{-6}$  M) (19, 20, 37), whereas it is unable to inhibit thrombin-catalyzed FXI activation (data not shown).

*Effects of Conformationally Constrained, A1-derived Peptides on the Activation of Factor XI by Thrombin*—Detailed structural information is not available for the A1 domain or any other part of the FXI molecule. However, we have constructed a molecular model of this region (20, 37), which may or may not ultimately be found to accurately portray the true structure of the FXI A1 domain once it is determined from x-ray crystallography or nuclear magnetic resonance studies. Thus, the model was used as a guide to generate hypotheses about the structure of the A1 domain to be tested in functional studies. Using this hypothetical model, we have made testable predictions about the structure and function of the HK binding site in the A1 domain (20, 37). Our experiments revealed that the sequence of amino acids, Val<sup>59</sup>-Lys<sup>83</sup> within the A1 domain of FXI, contains two antiparallel  $\beta$ -strands connected by  $\beta$ -turns that comprise a continuous surface utilized for the binding of HK (20). Since the rA1 domain peptide (Glu<sup>1</sup>-Ser<sup>90</sup>) was more effective in inhibiting FXI activation by thrombin than the peptide (Phe<sup>56</sup>-Ser<sup>86</sup>) by 2 orders of magnitude, we suspected that the amino acid sequence Glu<sup>1</sup>-Trp<sup>55</sup> might contain residues important in binding thrombin. Therefore, we examined a molecular model of the A1 domain that predicts the presence of three stem-loop structures (antiparallel  $\beta$ -strands connected by  $\beta$ -turns) defined by amino acid residues Ala<sup>45</sup>-Arg<sup>54</sup>, Val<sup>59</sup>-Arg<sup>70</sup>, and Asn<sup>72</sup>-Lys<sup>83</sup> (19, 20). We therefore prepared conformationally constrained cyclic peptides compris-

ing these peptide loop structures to determine whether they might assume a conformation that comprises a thrombin binding site. These peptides were identical to those tested to delineate the HK binding surface (19, 20, 37). Cysteine residues were introduced at the amino and carboxyl terminus of each peptide so that the resulting disulfide bond might stabilize the loop-like structure (19, 20). The peptide designated Ala<sup>45</sup>-Arg<sup>54</sup>(C) in which cysteines were substituted at positions 49 and 53 (see Table I) had no effect (refolded or reduced and alkylated) on FXI binding to HK at concentrations up to 10 mM (20). By comparison, this peptide (Ala<sup>45</sup>-Arg<sup>54</sup>) when properly folded inhibited thrombin-catalyzed activation of FXI with an  $IC_{50} \sim 2 \times 10^{-5}$  M. This peptide was 1 order of magnitude more effective than peptide F56-S86 ( $IC_{50} = 1 \times 10^{-4}$  M), which indicates that most of the binding energy for thrombin resides within this amino acid sequence (Ala<sup>45</sup>-Arg<sup>54</sup>) of FXI (Fig. 3A). The ability of Val<sup>59</sup>-Arg<sup>70</sup> (refolded) to inhibit thrombin-catalyzed FXI activation (Fig. 3B) was identical to that of Phe<sup>56</sup>-Ser<sup>86</sup> (Fig. 3A) with an  $IC_{50}$  of  $10^{-4}$  M. However, Asn<sup>72</sup>-Lys<sup>83</sup> (both refolded and reduced and alkylated) had no effect on thrombin-catalyzed activation of FXI (data not shown), whereas this peptide was a potent inhibitor of HK binding to FXI ( $IC_{50} \sim 10^{-4}$  M; Refs. 20 and 37). These results strongly suggest that Ala<sup>45</sup>-Arg<sup>54</sup> and Val<sup>59</sup>-Arg<sup>70</sup> form two stem-loop structures consisting of antiparallel  $\beta$ -strands connected by  $\beta$ -turns, which together form the thrombin binding site. The experiments in Fig. 3 demonstrate synergism in the effects of both peptides together compared with either one alone, since when the two peptides were added together their effect was greater than a simple additive effect, *i.e.* when used in combination at equimolar concentrations these two peptides were 1 order of magnitude more effective than either one alone in inhibiting thrombin-catalyzed FXI activation.

Prekallikrein, a protein with 58% sequence identity to FXI, also binds HK in the A1 domain within the homologous amino acid sequence Phe<sup>56</sup>-Gly<sup>86</sup> (38). This stretch of amino acids displays 65% homology with a comparable sequence in FXI. Therefore, we tested the PK Phe<sup>56</sup>-Gly<sup>86</sup> peptide for its ability to inhibit thrombin-catalyzed FXI activation. Unlike the FXI Phe<sup>56</sup>-Ser<sup>86</sup> peptide, the PK Phe<sup>56</sup>-Gly<sup>86</sup> peptide did not inhibit thrombin-catalyzed FXI activation (Fig. 3A). It has been reported that this sequence of amino acids in PK (Phe<sup>56</sup>-Gly<sup>86</sup>) binds HK (38) as does the homologous sequence of FXI (19, 20). Thus, the amino acid sequences involved in FXI and PK interaction with HK are not involved in binding thrombin.

**Fine Mapping of the Thrombin Binding Site in Factor XI**—In order to gain information about which specific amino acids comprise the thrombin binding site we prepared synthetic peptides with amino acid substitutions, determined by examining our molecular model for residues that project their side chains into a predicted contact surface (37). One such candidate in FXI was a glutamic acid residue at position 66 (37), which was therefore changed to an alanine in the conformationally constrained synthetic peptide Val<sup>59</sup>-Arg<sup>70</sup>. This altered peptide failed to inhibit thrombin-catalyzed FXI activation, whereas the native peptide with a glutamic acid present at position 66 (Val<sup>59</sup>-Arg<sup>70</sup>) inhibited thrombin-catalyzed FXI activation with an  $IC_{50}$  of  $\sim 10^{-4}$  M (Fig. 3B). However, Glu<sup>66</sup> is apparently not involved in binding HK since Val<sup>59</sup>-Arg<sup>70</sup> (Glu<sup>66</sup> → Ala) had inhibitory activity equal to that of the native peptide in FXI binding to HK (37). When the linear peptide Ala<sup>45</sup>-Arg<sup>54</sup> (Fig. 3C) was compared with the conformationally constrained cyclic peptide Ala<sup>45</sup>-Arg<sup>54</sup>(C) (Fig. 3B) they both inhibited thrombin-catalyzed FXI activation with the same  $IC_{50}$  ( $2 \times 10^{-5}$  M; see Table II), indicating 1) that Ser<sup>49</sup> and Thr<sup>53</sup> (which were substituted with cysteines in the cyclic peptide) are not

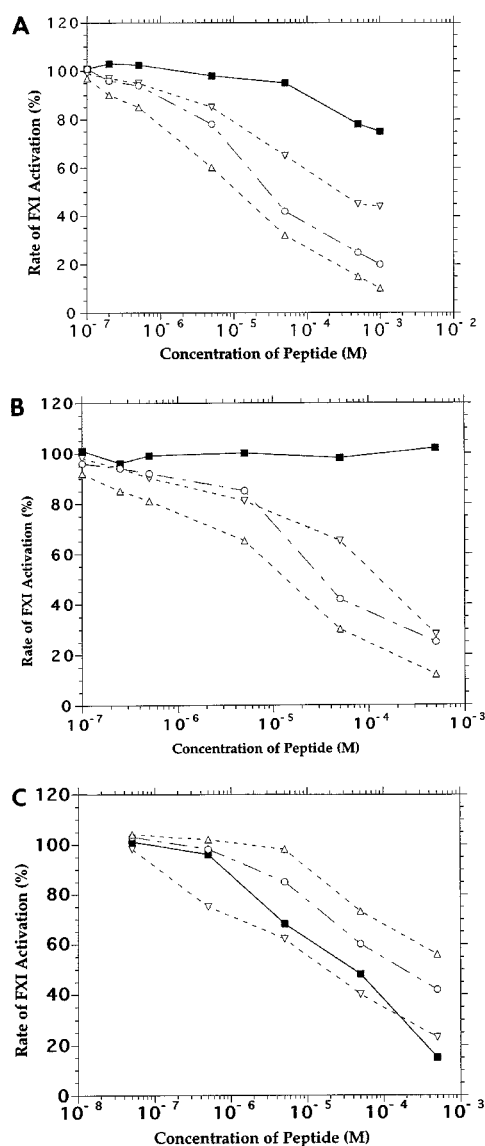


FIG. 3. Effects on the rate of activation of FXI (60 nM) by thrombin (1.25 nM) of the presence of various concentrations of heavy chain-derived peptides. Thrombin was incubated with either buffer or various concentrations of peptides prior to addition to the assay. The rate of FXIa formation was determined as described under "Materials and Methods." A, data shown are those with Phe<sup>56</sup>-Gly<sup>86</sup> (PK) (■—■), Phe<sup>56</sup>-Ser<sup>86</sup> (FXI) (▽—▽), Ala<sup>45</sup>-Arg<sup>54</sup>(C) (○—○), and the following peptides were added in combination at equimolar mixtures: Ala<sup>45</sup>-Arg<sup>54</sup>(C) plus Phe<sup>56</sup>-Ser<sup>86</sup> (△—△). B, data shown are those with Val<sup>59</sup>-Arg<sup>70</sup> (Glu<sup>66</sup> → Ala<sup>66</sup>) (■—■), Val<sup>59</sup>-Arg<sup>70</sup>(C) (▽—▽), Ala<sup>45</sup>-Arg<sup>54</sup>(C) (○—○), and the following peptides were added in combination at equimolar mixtures: Ala<sup>45</sup>-Arg<sup>54</sup>(C) plus V59-R70(C) (△—△). C, data shown are those with Ala<sup>45</sup>-Arg<sup>54</sup>(C) (Glu<sup>50</sup> → Ala) (■—■), Ala<sup>45</sup>-Arg<sup>54</sup> (▽—▽), Ala<sup>45</sup>-Arg<sup>54</sup>(C) (Asp<sup>51</sup> → Ala) (○—○), and Cys<sup>49</sup>-Cys<sup>53</sup>(C) (△—△).

part of the contact surface and 2) that cyclization and conformational constraint are not required for inhibitory activity of this particular peptide. However, when alanine was substituted for Asp<sup>51</sup> in peptide Ala<sup>45</sup>-Arg<sup>54</sup>(C) the altered peptide ( $IC_{50} = 1.5 \times 10^{-4}$  M) was only one-tenth as effective as the native peptide ( $IC_{50} = 2.0 \times 10^{-5}$  M) in inhibiting thrombin-catalyzed FXI activation. Therefore, Asp<sup>51</sup> may be an important constituent of the thrombin binding site. By comparison, the substitution at position 50 of an alanine for a glutamic acid in peptide Ala<sup>45</sup>-Arg<sup>54</sup>(C) resulted in only a modest (2.5-fold) decrease in inhibitory potency, with an  $IC_{50}$  of  $5 \times 10^{-5}$  M for

TABLE II  
Effects of recombinant and synthetic A1 domain peptides on  
thrombin-catalyzed activation of factor XI

Peptides	Concentration of peptide required to inhibit thrombin-catalyzed activation of FXI by 50%
Factor XI	<i>M</i>
Glu <sup>1</sup> -Ser <sup>90</sup>	$1.5 \times 10^{-6}$
Phe <sup>56</sup> -Ser <sup>86</sup>	$1.0 \times 10^{-4}$
Glu <sup>1</sup> -Ser <sup>90</sup> (Ile <sup>77</sup> → Ala <sup>77</sup> )	$2.0 \times 10^{-6}$
Glu <sup>1</sup> -Ser <sup>90</sup> (Val <sup>64</sup> → Ala <sup>64</sup> )	$2.5 \times 10^{-5}$
Ala <sup>45</sup> -Arg <sup>54</sup> (C)	$2.0 \times 10^{-5}$
Val <sup>59</sup> -Arg <sup>70</sup> (C)	$1.5 \times 10^{-4}$
Ala <sup>45</sup> -Arg <sup>54</sup> + Val <sup>59</sup> -Arg <sup>70</sup> (C) <sup>a</sup>	$0.2 \times 10^{-5}$
Ala <sup>45</sup> -Arg <sup>54</sup> (C) + Phe <sup>56</sup> -Ser <sup>86a</sup>	$0.1 \times 10^{-5}$
Ala <sup>45</sup> -Arg <sup>54</sup>	$1.5 \times 10^{-5}$
Ala <sup>45</sup> -Arg <sup>54</sup> (C) (Glu <sup>50</sup> → Ala <sup>50</sup> )	$5.0 \times 10^{-5}$
Ala <sup>45</sup> -Arg <sup>54</sup> (C) (Asp <sup>51</sup> → Ala <sup>51</sup> )	$1.5 \times 10^{-4}$
Glu <sup>50</sup> -Pro <sup>52</sup> (C)	$1.0 \times 10^{-3}$
Asn <sup>72</sup> -Lys <sup>83</sup>	NE <sup>b</sup>
Val <sup>59</sup> -Arg <sup>70</sup> (Glu <sup>66</sup> → Ala <sup>66</sup> )	NE <sup>b</sup>
Prekallikrein	
Phe <sup>56</sup> -Gly <sup>86</sup>	NE <sup>b</sup>
Pro <sup>45</sup> -Lys <sup>54</sup>	NE <sup>b</sup>

<sup>a</sup> Data are shown for equimolar mixture of two peptides.

<sup>b</sup> NE, no effect up to  $10^{-3}$  M.

the altered peptide compared with  $2 \times 10^{-5}$  M for the native peptide (Fig. 3C and Table II). Thus, these results indicate that the side chain of Asp<sup>51</sup> might be directly involved in binding thrombin, whereas Glu<sup>50</sup> might also make a minor contribution to thrombin binding. When the amino acids comprising part of the putative thrombin binding surface of Ala<sup>45</sup>-Arg<sup>54</sup>, consisting of amino acids Glu<sup>50</sup>-Asp<sup>51</sup>-Pro<sup>52</sup>, were tested as a conformationally constrained cyclic peptide, Ser<sup>49</sup>-Thr<sup>53</sup>(C), for its ability to inhibit thrombin-catalyzed FXI activation, it was 2 orders of magnitude less effective ( $IC_{50} = 1 \times 10^{-3}$  M; Fig. 3C and Table II) than the Ala<sup>45</sup>-Arg<sup>54</sup> peptide ( $IC_{50} = 2 \times 10^{-5}$  M; Fig. 3A and Table II). This result suggests that the full effect of the peptide Ala<sup>45</sup>-Arg<sup>54</sup> on thrombin-catalyzed FXI activation requires a contribution from the amino-terminal portion in addition to Ser<sup>49</sup>-Thr<sup>53</sup>.

**Kinetics of Thrombin-catalyzed Factor-XI Activation in the Presence of the Monoclonal 5F7 or the rA1 Domain Glu<sup>1</sup>-Ser<sup>90</sup>**—The activation of FXI by thrombin in the presence of various concentrations of either monoclonal antibody 5F7 or the rA1 domain (Glu<sup>1</sup>-Ser<sup>90</sup>) are shown in Fig. 4. The double-reciprocal (Lineweaver-Burk) plots yielded patterns consistent with classical competitive inhibition, *i.e.*  $V_{max}$  is unaffected by the antibody or peptide, whereas progressively higher concentrations of the antibody or peptide yielded progressively higher values of apparent  $K_m$ . These results suggest that the binding of the rA1 domain Glu<sup>1</sup>-Ser<sup>90</sup> to a putative substrate (FXI) binding site within the thrombin molecule reduces the effective concentration of this binding site for its substrate (FXI), whereas the catalytic site of thrombin remains unaltered. The 5F7 monoclonal antibody apparently binds to an epitope within the A1 domain close to the site that interacts with the putative thrombin substrate (FXI) binding site.

**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 under "Materials and Methods." In the case of competitive inhibition,  $I_{50}$  (the total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction) is a linear function of the substrate concentration. From the slope of this line  $K_i$  can be determined. The insets of Fig. 4, A and B, represent such a plot, with the monoclonal antibody 5F7 (Fig. 4A) or the rA1 domain (Glu<sup>1</sup>-Ser<sup>90</sup>) peptide (Fig. 4B) used as inhibitors. The calculated val-

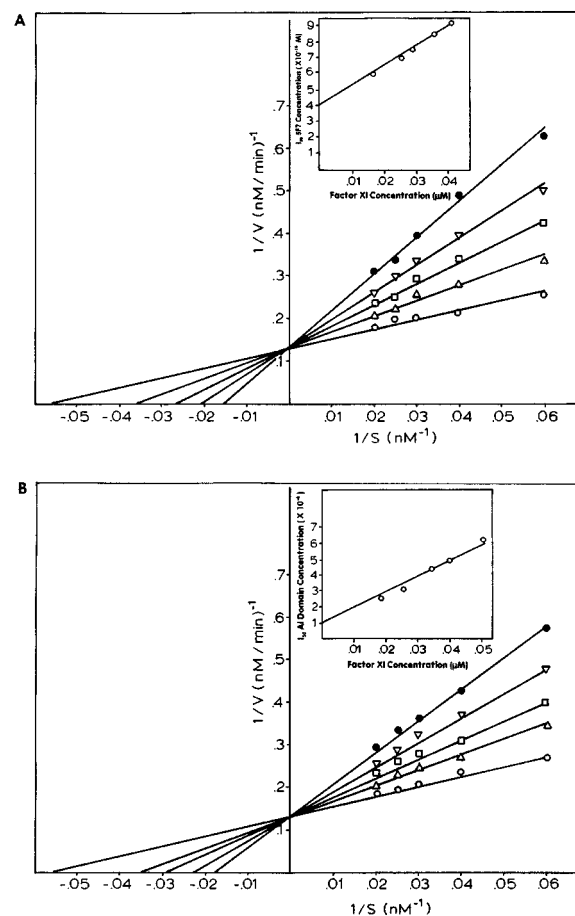


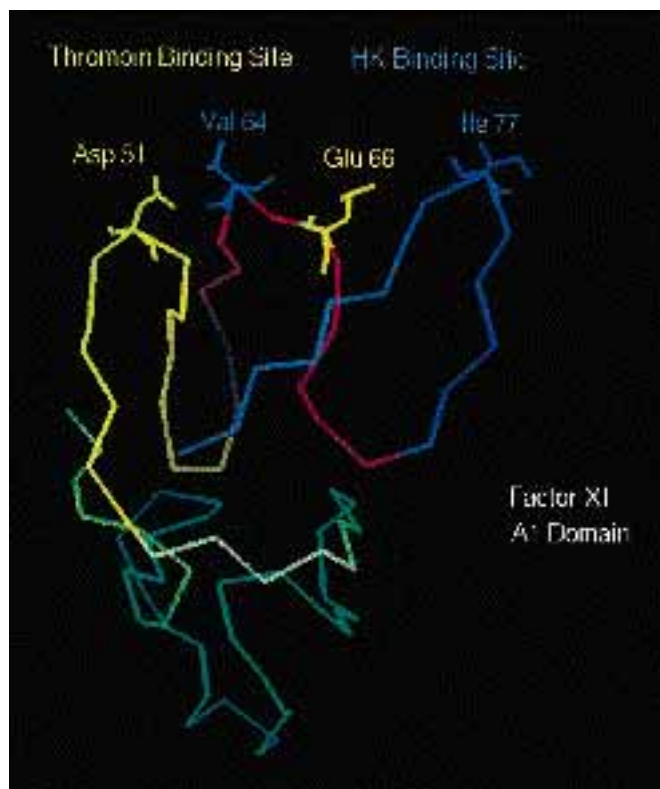
FIG. 4. Double-reciprocal plots of the activation of FXI by thrombin in the presence of various concentrations of 5F7 monoclonal antibody (A) and rA1 domain peptide Glu<sup>1</sup>-Ser<sup>90</sup> (B). A, FXI was incubated with various concentrations of antibody 5F7 at 37 °C and added to the assay buffer (TBBSA). Final concentration of thrombin was 25 nM. FXI was used in the range of 16–50 nM, and the rate of FXIa formation was determined as described under "Materials and Methods." The results shown are double-reciprocal plots in the absence (○—○) and in the presence of antibody at concentrations of  $3 \times 10^{-10}$  M (△—△),  $9 \times 10^{-10}$  M (□—□),  $1.2 \times 10^{-9}$  M (▽—▽), and  $1.8 \times 10^{-9}$  M (●—●). The inset is the secondary plot of  $I_{50}$  peptide concentration versus substrate concentration as described by Cha (35). B, thrombin (25 nM) was incubated with various concentrations of peptide for 15 min at 37 °C. FXI was used in the range of 16–50 nM as shown on the abscissa, and the rate of FXIa formation was determined as described under "Materials and Methods." The results are shown as double-reciprocal plots in the absence (○—○) and in the presence of peptide Glu<sup>1</sup>-Ser<sup>90</sup> at concentrations of  $2.8 \times 10^{-8}$  M (△—△),  $2.8 \times 10^{-7}$  M (■—■),  $2.8 \times 10^{-6}$  M (▽—▽), and  $2.8 \times 10^{-5}$  M (●—●). The inset is the secondary plot of  $I_{50}$  peptide concentration versus substrate concentration.

ues of  $K_i$  from the slopes are  $4 \times 10^{-10}$  M (5F7) and  $1 \times 10^{-6}$  M (rA1, Glu<sup>1</sup>-Ser<sup>90</sup>).

#### DISCUSSION

It is possible to activate FXI in the absence of contact proteins in the presence of the serine protease thrombin (15, 16, 18). Consequently, several laboratories have attempted to determine the physiological conditions required for the activation of FXI by thrombin or other proteases (16, 18, 39). Although FXI is activated by thrombin in a purified system, it is suspected that this reaction may not proceed in plasma (18, 39). It is well known that HK enhances FXIIa-mediated activation of FXI *in vitro* (1–10). However, HK (500 nM) inhibits thrombin-mediated activation of FXI (15, 16, 18, 39). Thus, thrombin may not be a suitable activator of FXI in plasma. To understand the physiological importance of thrombin-mediated FXI activation,





**FIG. 5. Molecular model of the A1 domain of FXI.** Using the primary structure of the A1 domain and its known disulfide linkages, a model was calculated demonstrating antiparallel  $\beta$ -strands connected by  $\beta$ -turns (20). The colors represent the peptide loop structures ( $\alpha$ -carbon backbone) and amino acids postulated to comprise binding sites for thrombin (yellow) (residues 45–58), HK (blue) (residues 72–85) and a loop structure utilized for binding both (or either) protein (red) (residues 59–71).

Our experiments support the conclusion that a sequence of amino acids (Ala<sup>45</sup>–Lys<sup>70</sup>) in the A1 domain of FXI that contains two antiparallel  $\beta$ -strands connected by  $\beta$ -turns comprises a surface that interacts with a substrate (FXI) binding site within thrombin (Fig. 5). The evidence supporting this conclusion is as follows: 1) a monoclonal antibody (5F7) that binds to the A1 domain of FXI (19, 20, 36) can completely block thrombin-catalyzed FXI activation with a  $K_i \sim 5 \times 10^{-10}$  M (close to the  $K_d$  for 5F7 binding to FXI; see Ref. 36 and Figs. 1 and 4); 2) the rA1 domain peptide (Glu<sup>1</sup>–Ser<sup>90</sup>) inhibited the activation of FXI by thrombin with a  $K_i$  of  $5 \times 10^{-6}$  M (Figs. 1 and 4); 3) a molecular model of the A1 domain (Fig. 5) predicts the presence of three peptide loop structures, Ala<sup>45</sup>–Arg<sup>54</sup>, Val<sup>59</sup>–Arg<sup>70</sup>, and Asn<sup>72</sup>–Lys<sup>83</sup>, that form a solvent-exposed surface (37); 4) based on this model, conformationally constrained peptides were synthesized, two of which (Ala<sup>45</sup>–Arg<sup>54</sup>, Val<sup>59</sup>–Arg<sup>70</sup>) act synergistically to inhibit thrombin-catalyzed FXI activation (Fig. 3); and, 5) Lineweaver-Burk plots of the activation of FXI by thrombin in the presence of either the monoclonal antibody 5F7 or the rA1 domain peptide yielded patterns consistent with a classical competitive inhibition (Fig. 4).

We have previously characterized a binding site for HK in the A1 domain of FXI (19, 20, 36, 37). To perform fine mapping of this site we prepared conformationally constrained synthetic peptides and rA1 domain constructs (20, 37). To identify specific amino acid residues involved in HK binding, conformationally constrained peptides were synthesized containing conserva-

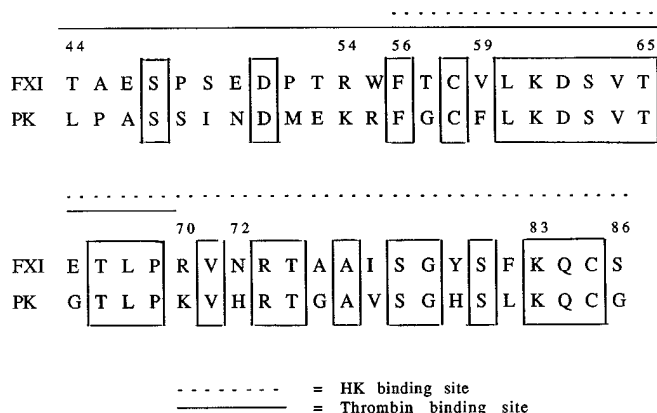


FIG. 6. **Comparison of amino acid sequences of portions of the Apple 1 domains of FXI and PK.** The positions that have identical residues are *boxed*. The primary structure of the A1 domain in FXI and PK (7) are *boxed*.

ative amino acid substitutions at residues suspected to contain side chains involved in binding including Val<sup>64</sup> → Ala, Glu<sup>66</sup> → A, Arg<sup>73</sup> → Ala, and Ile<sup>77</sup> → Ala (37). Because abnormal results were obtained with two of these peptides, Val<sup>64</sup> → Ala and Ile<sup>77</sup> Ala, which failed to compete normally with FXI for binding HK, we prepared two mutant rA1 domains (Val<sup>64</sup> → Ala and Ile<sup>77</sup> Ala), both of which exhibited diminished capacity to inhibit FXI binding to HK (37). Since the thrombin binding site was localized to the A1 domain and found to contain amino acid sequences overlapping the HK binding site (Fig. 1), we attempted to identify specific amino acid residues in the A1 domain that might bind thrombin. Our results are consistent with the following conclusions: 1) Val<sup>64</sup> and Ile<sup>77</sup>, which are important as contact sites for HK (37), do not participate in the interaction of the A1 domain with thrombin (Fig. 2); 2) Glu<sup>66</sup> and Asp<sup>51</sup>, which are not important as contact sites for binding HK (37), are both apparently important residues for binding thrombin (Fig. 3); and 3) another important difference between the HK and thrombin binding sites in the A1 domain is that reduction and alkylation of the A1 domain virtually destroys the thrombin binding site while leaving the HK binding site intact (37).

We also examined the plasma protein PK, which shares a high degree (58%) of sequence identity with FXI (7), to determine whether homologous amino acid sequences can also inhibit thrombin-catalyzed FXI activation. Unlike the FXI Phe<sup>56</sup>-Ser<sup>86</sup> peptide, the PK Phe<sup>56</sup>-Gly<sup>86</sup> peptide did not inhibit thrombin-catalyzed FXI activation. A glutamic acid is replaced by a glycine at position 66 in PK, and this amino acid substitution Glu<sup>66</sup> → Ala<sup>66</sup> in FXI Val<sup>59</sup>-Arg<sup>70</sup> rendered this peptide inactive (Fig. 3). It is possible that this and other amino acid replacements in PK render PK Phe<sup>56</sup>-Gly<sup>86</sup> unable to inhibit thrombin-catalyzed FXI activation. It has also been established that PK, like FXI, also binds HK in the A1 domain within the homologous amino acid residues Phe<sup>56</sup>-Gly<sup>86</sup> (38). This stretch of amino acids is 65% identical to the comparable sequence in FXI (Fig. 6). Moreover, a peptide with the amino acid sequence Pro<sup>45</sup>-Lys<sup>54</sup> of PK had no effect on thrombin-catalyzed FXI activation, unlike Ala<sup>45</sup>-Arg<sup>54</sup> of FXI, which was the most effective inhibitor (Fig. 3 and Table II). These two regions of FXI and PK have only 18% identity (Fig. 6), and it seems reasonable that PK does not contain a binding site for thrombin since thrombin does not activate PK (40). We have also attempted to fine map the important contact sites of peptide Ala<sup>45</sup>-Arg<sup>54</sup>(C) that interacts with thrombin. Apparently, Asp<sup>51</sup> is important in this interaction. However, amino acid residues in the first half of the peptide Ala<sup>45</sup>-Pro<sup>48</sup> also appear to be important in its interaction with thrombin (Fig. 3).

The data presented in this paper support the conclusion that the thrombin and HK binding sites in the A1 domain, while contiguous, are separate and distinct. However, these two binding sites appear to overlap since they share a common sequence of amino acids (Val<sup>59</sup>–Arg<sup>70</sup>). The relationship between the putative thrombin and HK binding sites is depicted in the molecular model shown in Fig. 5. The model and our results predict that if HK is bound to the A1 domain, thrombin-mediated activation of FXI would be blocked and FXIIa-mediated activation of FXI would be favored. The reverse may also occur, *i.e.* the binding of thrombin to the A1 domain should prevent HK binding and FXIIa-mediated activation of FXI. Therefore, these two contiguous partially overlapping sites could constitute a point of regulation to determine by which pathway (contact activation *versus* feedback activation) and by which protease (FXIIa or thrombin) FXI might be activated.

**Acknowledgments**—We are grateful to Patricia Pileggi for assistance in manuscript preparation.

#### REFERENCES

- Davie, E. W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **48**, 277–318
- Thompson, R. E., Mandle, R., and Kaplan, A. P. (1977) *J. Clin. Invest.* **60**, 1376–1380
- Griffin, J. H., and Cochrane, C. G. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 2554–2558
- Bouma, P. N., and Griffin, J. H. (1977) *J. Biol. Chem.* **252**, 6432–6437
- Kurachi, K., Fujikawa, K., and Davie, E. W. (1980) *Biochemistry* **19**, 1330–1338
- Ohkubo, I., Fujikawa, K., and Kurachi, K. (1982) *Fed. Proc. Am. Soc. Exp. Biol.* **41**, 656 (abstr.)
- Fujikawa, K., Chung, D. W., Hendrickson, L., and Davie, E. W. (1986) *Biochemistry* **25**, 2417–2424
- McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) *Biochemistry* **30**, 2056–2060
- van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M., and Griffin, J. H. (1983) *J. Biol. Chem.* **258**, 9669–9675
- Kurachi, K., and Davie, E. W. (1977) *Biochemistry* **16**, 5831–5839
- Revak, S. D., Cochrane, C. G., and Griffin, J. H. (1977) *J. Clin. Invest.* **59**, 1167–1175
- Schmaier, A. H., Silverberg, M., Kaplan, A. P., and Colman, R. W. (1987) in *Hemostasis and Thrombosis*, 2nd Ed., (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 18–38, Lippincott, Philadelphia, PA
- Ragni, M. V., Sinha, D., Seaman, F., Lewis, J. H., Spero, J. A., and Walsh, P. N. (1985) *Blood* **65**, 719–724
- Bolton-Maggs, P. H. B., Young Wan-Yin, R., McCraw, A., Slack, J., and Kernoff, P. B. A. (1988) *Br. J. Haematol.* **69**, 521–528
- Naito, K., and Fujikawa, K. (1991) *J. Biol. Chem.* **266**, 7353–7358
- Gailani, D., and Broze, G. J. (1991) *Science* **253**, 909–912
- Mann, K. G., and Lundblad, R. L. (1987) in *Hemostasis and Thrombosis*, 2nd Ed., (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 148–161, Lippincott, Philadelphia, PA
- Scott, C. F., and Colman, R. W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 11189–11193
- 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. (1992) *J. Biol. Chem.* **267**, 4247–4252
- Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1993) *J. Biol. Chem.* **268**, 3838–3844
- Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1991) *J. Biol. Chem.* **266**, 24190–24197
- Baglia, F. A., Jameson, B. A., and Walsh, P. N. (1995) *J. Biol. Chem.* **270**, 6734–6740
- Sinha, D., Koshy, A., Seaman, F. S., and Walsh, P. N. (1985) *J. Biol. Chem.* **260**, 10714–10719
- Page, J. D., and Colman, R. W. (1991) *J. Biol. Chem.* **266**, 8143–8148
- Scott, C. F., Sinha, D., Seaman, F. S., Walsh, P. N., and Colman, R. W. (1984) *Blood* **63**, 42–50
- Proctor, R. R., and Rapaport, S. I. (1961) *Am. J. Clin. Pathol.* **36**, 212–219
- Kent, S. B. H., and Clark-Lewis, I. (1985) *Synthetic Peptides in Biology and Medicine*, Elsevier Science Publishers, Amsterdam
- Walsh, P. N., Baglia, F. A., and Jameson, B. A. (1993) *Methods Enzymol.* **222**, 65–96
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
- Budzynski, A. Z., Olexa, S. A., and Brizuela, B. S. (1979) *Biochim. Biophys. Acta* **584**, 284–287
- Segal, I. H. (1974) *Enzyme Kinetics*, Wiley-Interscience, New York, NY
- Sinha, D., Seaman, F. S., and Walsh, P. N. (1987) *Biochemistry* **26**, 3768–3775
- Walsh, P. N., Bradford, H., Sinha, D., Piperno, J. R., and Tuszyński, G. P. (1984) *J. Clin. Invest.* **73**, 1392–1399
- Cha, S. (1975) *Biochem. Pharmacol.* **24**, 2177–2185
- Baglia, F. A., Sinha, D., and Walsh, P. N. (1989) *Blood* **74**, 244–251
- Seaman, F. S., Baglia, F. A., Gurr, J. A., Jameson, B. A., and Walsh, P. N. (1994) *Biochem. J.* **304**, 715–721
- Herwald, H., Jahnen-Dechent, W., Abd Alla, S. A., Hock, J., Bouma, B., and Muller-Esterl, W. (1993) *J. Biol. Chem.* **268**, 14527–14535
- Brunnee, T., LaPorta, C., Reddigari, S. R., Salerno, V. M., Kaplan, A. P., and Silverberg, M. (1993) *Blood* **81**, 580–586
- Gailani, D., and Broze, S. W. (1993) *Sem. Thromb. Hemostasis* **19**, 396–404