Previously we defined binding sites for high molecular weight kininogen (HK) and thrombin in the Apple 1 (A1) domain of factor XI (FXI). Since prothrombin (and Ca²⁺) can bind FXI and can substitute for HK (and Zn²⁺) as a cofactor for FXI binding to platelets, we have attempted to identify a prothrombin-binding site in FXI. The recombinant A1 domain (rA1, Glu¹–Ser⁹⁰) inhibited the saturable, specific and reversible binding of prothrombin to FXI, whereas neither the rA2 domain (Ser⁷⁰–Ala¹⁸¹), rA3 domain (Ala¹⁸¹–Val²³¹), nor rA4 domain (Phe²⁷²–Glu³⁶¹) inhibited prothrombin binding to FXI. Kinetic binding studies using surface plasmon resonance showed binding of FXI (K_d = 71 nM) and the rA1 domain (K_d = 239 nM) but not rA2, rA3, or rA4 to immobilized prothrombin. Reciprocal binding studies revealed that synthetic peptides (encompassing residues Ala⁴⁵–Ser⁸⁶) containing both HK- and thrombin-binding sites, inhibit ¹²⁵I-rA1 (Glu¹–Ser⁹⁰) binding to prothrombin, ¹²⁵I-prothrombin binding to FXI, and ¹²⁵I-prothrombin fragment 2 (Ser¹⁵⁶–Arg²³¹) binding to FXI. However, homologous prekallikrein-derived peptides (encompassing Pro⁴⁸–Gly⁶⁶) did not inhibit FXI rA1 binding to prothrombin. The peptides Ala⁴⁵–Arg³⁴, Phe⁵⁶–Val⁷¹, and Asp⁷²–Ser⁸⁶, derived from sequences of the A1 domain of FXI, acted synergistically to inhibit ¹²⁵I-rA1 binding to prothrombin. Mutant rA1 peptides (V64A and 177A), which did not inhibit FXI binding to HK, retained full capacity to inhibit rA1 domain binding to prothrombin, and mutant rA1 peptides Ala⁴⁵–Ala⁵⁴ (D51A) and Val⁵⁶–Arg⁷⁰ (E66A), which did not inhibit FXI binding to thrombin, retained full capacity to inhibit rA1 domain binding to prothrombin. Thus, these experiments demonstrate that a prothrombin binding site exists in the A1 domain of FXI spanning residues Ala⁴⁵–Ser⁸⁶ that is contiguous with but separate and distinct from the HK- and thrombin-binding sites and that this interaction occurs through the kringle II domain of prothrombin.

Factor XI (FXI), a protein that participates in the intrinsic pathway of blood coagulation, consists of two identical polypepti-
assayed by a minor modification (22) of the kaolin-activated partial thromboplastin time. The recombinant Apple 1 (rA1) domain (Glu(1–
Ser(90)) was prepared and characterized as described previously (15, 18). Human prothrombin, PF1 and PF1.2 were purchased from Hema-
tologic Technologies, Inc. (Essex Junction, VT). HK (specific activity, 15
units/mg) was prepared and purified by the method of Stevens et al. (21). Human α-thrombin (2,800 NIH units/mg) was purchased from Enzyme
Research Laboratories (South Bend, IN). The potent thrombin inhibitor
n-Phe-Pro-Arg chloromethyl ketone (PPACK) was purchased from Cal-
biochem (Indianapolis, IN). Active site-inhibited thrombin was pre-
pared by incubation of a 10-fold excess of PPACK with α-thrombin for 1 h at 37 °C, and this mixture was subjected to a carboxylase reaction at
2,500 cut-off, Spectrum Medical Industries, Los Angeles, CA) overnight
in phosphate-buffered saline at 5 °C. All purified proteins appeared
homogeneous by SDS-polyacrylamide gel electrophoresis.

Isolation and Purification of Prothrombin Fragment 2 (PF2) from
Prothrombin—PF2 was isolated from prothrombin activation material
depleted of thrombin by SP chromatography (the source of this material
was from Enzyme Research Laboratories, South Bend, IN). This prep-
paration contained 0.4 mg/ml protein in 50 mM sodium phosphate and 1
μM benzamidine, pH 6.5. To 50 ml of this material, PFAPC (from Cal-
biochem, Indianapolis, IN) was added to a concentration of 10 μM.
PF2 was isolated utilizing a fast protein liquid chromatography system
(Amersham Pharmacia Biotech) at 23 °C using a MonoQ (HR 5/5) col-
mn, eluting according to a 4 procedure (22). PF2 was separated
in two separate pools, with PF2 migrating at 14 kDa as a doublet on
SDS-gel electrophoresis. From 50 ml (20 mg) of protein loaded on the
column, approximately 7 mg of PF2 was recovered. An additional prep-
paration of human PF2 was a generous gift from Dr. S. Krishnaswamy
(University of Pennsylvania, Philadelphia, PA) and gave identical re-
sults in all experiments reported here.

Radiolabeling of Proteins—Purified FXI, prothrombin, rA1 (Glu(1–
Ser(90)), and PF2 (Ser(165–Arg(271)) were radiolabeled with 125I by a
minor modification (21) of the IODOGEN method to a specific activity of 5 ×
10⁶ cpm/μg for FXI, 2.5 × 10⁶ cpm/μg for prothrombin, 1.0 × 10⁶ cpm/μg
for PF1.2, 0.5 × 10⁶ cpm/μg for rA1, and 0.25 × 10⁶ cpm/μg for PF2. The
radiola beled FXI and prothrombin retained >98% of their biological activity.

Protein Analysis—Protein concentrations were determined by the Bio-
Rad dye-binding assay according to the instructions provided by the
manufacturer.

Peptide Synthesis—Peptides were synthesized on an Applied Biosys-
tems 430A peptide synthesizer by a modification of the procedure de-
scribed by Kent and Clark-Lewis (25) and purified to >99% purity using
reverse phase high performance liquid chromatography (HPLC). The
sequences of the synthetic peptides utilized in this study have been
published previously (16–20). All the peptides utilized in this work
were rationally designed, conformationally constrained synthetic pep-
tides based upon previously published (16–20) molecular models for A1,
A2, A3, and A4 domains of FXI. A previously published method (26) was
used to oxidize the two cysteine residues in each peptide to form a disul-
fi de bond and to conformationally constrain the peptide.

HPLC—The HPLC system employed was from Waters (600 gradient
module, model 740 data module, model 46K universal injector, and
Lambda-Max model 481 detector; Waters, Milford, MA). Reverse phase
chromatography was performed using a Waters C8 μBondapak column,
whereas gel filtration was carried using a Waters Protein-Pak 60
column as described previously (26).

Characterization of Synthetic Peptides—All the peptides utilized in
this study were examined by HPLC (both reverse phase and gel fil-
tration), and all demonstrated a single homogeneous peak (data not
shown). When the 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.
All peptides were examined for free SH groups using the Ellman reagent
(5,5′-dithiobis(2-nitrobenzoic acid)). It was determined (27) that there
was less than 0.02 mol of free SH/mmol of peptide, which further verifies
that these peptides were homogeneous preparations consisting of in-
trinsically folded peptide.

Binding of 125I-Labeled Prothrombin, Prothrombin Fragment 2, or
Prothrombin Fragment 1.2 to Factor XI—The binding of prothrombin,
PF1, or PF1.2 to FXI was studied using polyvinyl chloride microtiter
plates, the wells of which were coated with FXI by incubation with 100
μl of protein (100 μg/ml) overnight at 4 °C. After residual binding sites
in the wells were blocked with bovine serum albumin for 2 h at 25 °C, 100
μl of 125I-prothrombin, 125I-PF1, or 125I-PF1.2 (50–3,000 nM) was
added to the wells and incubated for 3–4 h at room temperature. The
wells were thoroughly washed with phosphate-buffered saline/bovine
serum albumin and dried and counted in a 1470 Wallac Wizard γ
counter (Wallac Inc., Gaithersburg, MD).

Binding of 125I-Recombinant Apple 1 (Glu(1–Ser(90)) Domain to Pro-
thrombin—The binding of the rA1 domain of FXI to prothrombin
was studied using polyvinyl chloride microtiter plates, the wells of which
were coated with prothrombin by incubation with 100 μl of protein (100
μg/ml) overnight at 4 °C. After residual binding sites in the wells were
blocked with bovine serum albumin for 2 h at 25 °C, 100 μl of 125I-rA1
(Glu(1–Ser(90)) (50–3,000 nM) was added to the wells and incubated for
2 h at 37 °C. After washing, the wells were thoroughly washed with
phantospe-buffered saline/bovine serum albumin and dried and counted
in a 1470 Wallac Wizard γ counter.

Binding of Factor XI and Recombinant Apple 1 Domain to Prothrom-
bin on Surface Plasmon Resonance—Binding studies were performed
on a Biacore 2000 Flow Biosensor (Biacore, Inc., Uppsala, Sweden).
Prothrombin was immobilized on a carboxymethyl dextran (CM5) flow
cell surface using either aldehyde linkage of oxidized carbohydrate (on
prothrombin) or amine-coupling chemistry. For aldehyde linkage, pro-
thrombin was diluted to 1 mg/ml in 100 mM sodium acetate buffer, pH 5.5,
and placed on ice. After addition of sodium metaperiodate (1 mM), the
protein was desalted on a PD10 column that had been pre-equilibrated
in 10 mM sodium acetate, pH 4.0. The CM5 flow cell surface was activated
with a 5-min pulse of 1.15 mg of N-hydroxysuccinimide mixed with 7.5
mg of N-(3-dimethylaminopropyl)carbodiimide hydrochloride at 5 μl/
m. The surface was then hydrazide-modified with a 7-min pulse of 5
ml carbohydrates in water. Non-reacted sites were blocked with a
7-min pulse of 1 mM ethanolamine, pH 8.5. The oxidized prothrombin,
~10 μg/ml in sodium acetate, pH 4.0, was injected to a response level of
~150 response units. The flow rate was lowered to 2 μl/min. Cyanoboro-
hydride was injected for 20 min, followed by three 1-min injections of
0.1 mM glycine. For amine coupling, a 7-min pulse of N-hydroxysuccin-
imide/N-(3-dimethylaminopropyl)carbodiimide hydrochloride was fol-
lowed by injection of prothrombin, ~10 μg/ml in sodium acetate buffer,
pH 4.5, to a response level of ~500 response units. Any remaining
derivatized carboxymethyl groups were blocked by a 7-min injection of
1 mMethanolamine. Nonspecific binding was determined by protein bind-
ing to a derivatized and blocked flow cell with an unrelated antibody
bound. Wild type FXI was studied using both the aldehyde-linked prothrombin and amine-coupled prothrombin. Recombinant Apple 1
domain was studied on the amine-coupled prothrombin flow cell alone.
Serial dilutions of wild type FXI and recombinant Apple domains in
Hepes-buffered saline (HBS; 10 mM Heps, 150 mM NaCl, 0.005% surfactant P20, 5 mM CaCl₂), were injected with a 6-min association time
and 5-min dissociation time. After subtraction of nonspecific binding
curves, the association and dissociation rate constants were determined
using a global fit to a one to one Langmuir association model on
Biaevaluation software (Biacore, Inc.). The best fit was determined by a
χ² value of less than 10 or less than 5% of the equilibrium response unit
value. The χ² value is the square of the differences between the theoretical ideal curve and the actual curve was and was calculated according to Equation 1.

\[
\chi^2 = 2(n - r_f^2) / r_f
\]

where \( n \) indicates the number of data points, and \( p \) indicates the fitted parameters.

RESULTS

Binding of 125I-Prothrombin to Factor XI—Since contiguous
but distinct binding sites for HK (Val(392–Lys(398)) and thrombin
(Ala(464–Arg(468)) reside within the A1 domain of FXI (18, 20), we
previously examined the effects of prothrombin and its frag-
ments on the binding of FXI to HK (14). Our published studies
(14) show that active site-inhibited thrombin, prothrombin,
and PF1.2 all inhibit the binding of FXI to HK, whereas PF1
had no such effect. We therefore hypothesized that prothrombin may bind
FXI at or near the HK binding site in the A1 domain (Phe(96–Ser(106)). (14) These experiments suggest that the kringle II domain of prothrombin may interact with the A1
domain of FXI at a site close to but distinct from the thrombin-
binding site. We therefore investigated whether prothrombin
can interact with the Apple domains of FXI by examining
only with the rA1 domain (Glu1–Ser90) with a calculated $K_D = 239 \pm 83 \text{ nM}$ (Fig. 2B, Table II). In contrast neither the rA2 domain (Ser90–Ala181), nor the rA3 domain (Ala181–Val271), nor the rA4 domain (Phe272–Glu361) was able to bind prothrombin using surface plasmon resonance (Table II). Thus, these experiments reveal that a binding site for prothrombin exists in the A1 domain of FXI.

We next examined the effects of active site-inhibited (PPACK-treated) thrombin, PF1, and PF1.2 on the binding of prothrombin to FXI. The experiment presented in Fig. 1B and Table I demonstrates that PF1.2 inhibits the binding of prothrombin to FXI (with an $IC_{50} = 1.0 \times 10^{-6} \text{ M}$). PPACK-treated thrombin also displaced FXI from prothrombin ($IC_{50} = 5.0 \times 10^{-6} \text{ M}$), whereas PF1 had no effect. This experiment suggests that both prothrombin (utilizing the kringle II domain) as well as thrombin bind to the A1 domain of FXI.

The Binding of Factor XI Apple 1 Domain to Prothrombin—Since our experiments demonstrate that prothrombin can bind FXI and the rA1 (Glu1–Ser90) inhibits this interaction (Fig. 1A), we therefore determined whether the rA1 (Glu1–Ser90) binds prothrombin directly. Therefore, we performed experiments in which prothrombin was bound to the wells of a microtiter plate and determined whether 125I-rA1 (Glu1–Ser90) could bind to this protein. Our results indicate that 125I-rA1 (Glu1–Ser90) binds prothrombin in a saturable manner with a $K_{d(app)}$ of $471 \pm 175 \text{ nM}$ (Fig. 3 and Table I).

The Effect of Apple 1-derived Peptides on the Binding of Recombinant Apple 1 (Glu1–Ser90) to Prothrombin—The true structure of the FXI A1 domain is not known, but we have constructed a molecular model of this region (18). Using this model we have made predictions about the structure of the HK and thrombin binding sites (15, 18, 29). The peptides designated Ala45–Arg54 (thrombin binding site) and Phe56–Ser76 (HK binding site) both inhibited the binding of 125I-rA1 (Glu1–Ser90) to prothrombin with similar $IC_{50}$ values of $2.0 \times 10^{-4} \text{ M}$ and $7.0 \times 10^{-5} \text{ M}$, respectively (Fig. 4A, Table I), suggesting that both amino acid sequences common to thrombin and HK binding sites may bind prothrombin. When both peptides were added together at equimolar concentrations, they were 1 order of magnitude more effective (demonstrating synergism) than either one alone in inhibiting 125I-rA1 (Glu1–Ser90) binding to prothrombin (IC50 values of $2.0 \times 10^{-4} \text{ M}$ and $7.0 \times 10^{-5} \text{ M}$, respectively (Fig. 4A, Table I)), suggesting that the three peptide loop structures to determine whether they might assume a conformation that comprises a prothrombin binding site. These peptides were identical to those tested to delineate the HK and thrombin binding sites (15, 18, 29). The peptides designated Ala45–Arg54 (thrombin binding site) and Phe56–Ser76 (HK binding site) both inhibited the binding of 125I-rA1 (Glu1–Ser90) to prothrombin with similar $IC_{50}$ values of $2.0 \times 10^{-4} \text{ M}$ and $7.0 \times 10^{-5} \text{ M}$, respectively (Fig. 4A, Table I), suggesting that both amino acid sequences common to thrombin and HK binding sites may bind prothrombin. When both peptides were added together at equimolar concentrations, they were 1 order of magnitude more effective (demonstrating synergism) than either one alone in inhibiting FXI rA1 binding to prothrombin (Table I). Moreover, the three peptides, Ala45–Arg54, Asn72–Ser86, and Phe56–Val71, were effective inhibitors of rA1 binding to prothrombin with similar $IC_{50}$ values of $2.0 \times 10^{-4} \text{ M}$ (Fig. 4B, Table I). When combinations of equimolar concentrations of these peptides were added to this assay, an even greater effect was observed ($IC_{50} = 2.0 \times 10^{-6} \text{ M}$), demonstrating synergism in the effects of the peptides since when the peptides were added together their effect was greater than a simple additive effect (i.e. the mixture of peptides was 2 orders of magnitude more effective than either one alone in inhibiting 125I-rA1 domain binding to prothrombin). However, the three individual peptides, Ala45–Arg54, Asn72–Ser86, and Phe56–Val71, added to
Lys54) and PK (Phe56–Gly86) peptides for their ability to inhibit prothrombin. The PK peptides did not inhibit FXI rA1 binding to prothrombin, while the PK peptides did not inhibit FXI rA1 binding to prothrombin (IC50 5–10^{-5}).

Table I

| Assay                     | Protein       | Competing peptide (domain) or fragment | IC50 | Kd  |
|---------------------------|---------------|----------------------------------------|------|-----|
| Prothrombin binding to factor XI | Factor XI Glu1–Ser50 (rA1) | 2.5 ± 0.50 x 10^{-7} | 4 x 10^{-7} |
| Prothrombin binding to factor XI | Factor XI Ser50–Ala11 (rA2) | NE | NE |
| Prothrombin binding to factor XI | Factor XI Ala11–Val57 (rA3) | NE | NE |
| Prothrombin binding to factor XI | Factor XI Phe77–Glu96 (rA4) | NE | NE |
| Prothrombin binding to factor XI | Prothrombin | PF1 | 1 x 10^{-6} |
| Prothrombin binding to factor XI | Prothrombin | PF1.2 | 5 x 10^{-6} |
| PF1.2 binding to factor XI | Factor XI Phe56–Ser66 (A1) | 4.49 ± 1.72 x 10^{-7} | 5 x 10^{-5} |
| PF1.2 binding to factor XI | Factor XI Ala45–Arg54 (A1) | 6 x 10^{-5} |
| PF1.2 binding to factor XI | Prothrombin | Ser156–Arg171 (PF2) | 2 x 10^{-7} |
| rA1 binding to prothrombin | Factor XI Ser49–Thr53 (A1) | 4.71 ± 1.75 x 10^{-7} | NE |
| rA1 binding to prothrombin | Factor XI Ala45–Arg54 (E50A) (A1) | 2 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Ala45–Arg54 (D51A) (A1) | 1 x 10^{-4} |
| rA1 binding to prothrombin | Prekallikrein Pro45–Lys54 (A1) | NE |
| rA1 binding to prothrombin | Prekallikrein Phe56–Gly86 (A1) | NE |
| Prothrombin binding to factor XI | Factor XI Ala45–Arg54 (A1) | 2 x 10^{-4} |
| Prothrombin binding to factor XI | Factor XI Phe56–Ser66 (A1) | 7 x 10^{-5} |
| Prothrombin binding to factor XI | Factor XI Ala45–Arg54 + Phe56–Ser66 (A1) | 1.5 x 10^{-6} |
| Prothrombin binding to factor XI | Factor XI Glu1–Ser50 (rA1) | 3 x 10^{-7} |
| rA1 binding to prothrombin | Factor XI Asn72–Ser86 (A1) | 6 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Asn72–Lys83 (R73A) (A1) | 5 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Phe56–Arg70 (A1) | 2 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Val59–Arg70 (E66A) (A1) | 4 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Glu1–Ser50 (V64A) (A1) | 4 x 10^{-7} |
| rA1 binding to prothrombin | Factor XI Glu1–Ser50 (I77A) (A1) | 5 x 10^{-6} |
| rA1 binding to prothrombin | Factor XI Asn72–Ser86 (A1) | 2 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Phe56–Val71 (A1) | 2 x 10^{-4} |
| rA1 binding to prothrombin | Factor XI Phe56–Val71 + Asn72–Ser86 (A1) | 1.5 x 10^{-5} |
| rA1 binding to prothrombin | Factor XI Ala45–Arg54 + Phe56–Val71 (A1) | 7.5 x 10^{-6} |
| rA1 binding to prothrombin | Factor XI Ala45–Arg54 + Asn72–Ser86 (A1) | 2 x 10^{-6} |
| PF2 binding to factor XI | Factor XI Ala45–Arg54 (A1) | 4.17 ± 1.9 x 10^{-7} | 4 x 10^{-4} |
| PF2 binding to factor XI | Factor XI Phe56–Ser66 (A1) | 5 x 10^{-5} |
| PF2 binding to factor XI | Prothrombin | Ser156–Arg171 (PF2) | 2 x 10^{-7} |
| PF2 binding to factor XI | Factor XI Phe56–Val71 (A1) | 5 x 10^{-5} |
| PF2 binding to factor XI | Factor XI Asn72–Ser86 (A1) | 5 x 10^{-5} |
| PF2 binding to factor XI | Factor XI Ala45–Arg54 + Phe56–Val71 (A1) | 6 x 10^{-5} |
| PF2 binding to factor XI | Factor XI Ala45–Arg54 + Asn72–Ser86 (A1) | 3 x 10^{-5} |
| PF2 binding to factor XI | Factor XI Phe56–Val71 + Asn72–Ser86 (A1) | 2.5 x 10^{-5} |
| PF2 binding to factor XI | Factor XI Ala45–Arg54 + Phe56–Val71 + Asn72–Ser86 (A1) | 3 x 10^{-6} |

NE, no effect up to 10^{-5} M.

Previously, in order to gain information about the HK and thrombin binding sites in the A1 domain of FXI, we prepared synthetic peptides with amino acid substitutions, determined by examining a molecular model for residues that project their side chains into a predicted contact surface (15, 29). We have identified specific amino acid residues within the A1 domain involved in binding thrombin (15). We have determined that Glu56 and Asp51 of the A1 domain of FXI are important components of the thrombin binding site in FXI (15). However, the altered peptides Val59–Arg70 (E66A) and Ala45–Arg54 (D51A) inhibited the binding of the rA1 domain to prothrombin (IC50 = 4 x 10^{-4} M and 1 x 10^{-4} M, respectively) with IC50 values similar to that of the wild-type peptides (Fig. 5 (A and B), Table I). Thus, prothrombin does not bind the A1 domain of FXI through amino acid sequences found to be important for thrombin binding to the A1 domain (15). By comparison, substitutions at position 50 of an alanine for glutamic acid in peptide Ala45–Arg54 and at position 73 of an alanine for arginine in peptide Asn72–Lys83 did not decrease the inhibitory potency (Fig. 5 (A and B), Table I). Utilizing mutational analysis, we

gathered less effective (IC50 = 2 x 10^{-6} M) in inhibiting 125I-rA1 binding to prothrombin than the rA1 domain (IC50 = 4 x 10^{-7} M) (Fig. 4 (A and B) and Table I). This may be due to a loss in conformational structure of the individual peptides. Thus, these experiments suggest that the binding site for prothrombin in the A1 domain of FXI is defined by the molecular model of the A1 domain that predicts the presence of three stem-loop structures defined by residues Ala45–Lys83 (15, 18, 29).

Prekallikrein (PK) is a protein that shares a 58% sequence identity with FXI (4). It also binds HK in the A1 domain within the homologous amino acids Phe56–Gly86 (29), but does not bind thrombin (15, 30). Therefore, we tested the PK (Pro45–Lys54) and PK (Phe56–Gly86) peptides for their ability to inhibit FXI rA1 binding to prothrombin. Unlike the corresponding FXI peptides, the PK peptides did not inhibit FXI rA1 binding to prothrombin (Fig. 4A, Table I). Thus, the amino acid sequences involved in PK interactions with HK are not involved in binding prothrombin.

Mapping of the Prothrombin Binding Site in Factor XI—
Prothrombin Kringle II Domain Binds to FXI Apple 1 Domain

Prothrombin Fragment 1.2 or Prothrombin Fragment 2 to Factor XI—Fig. 1 and Table I show that the rA1 domain inhibits the binding of prothrombin to FXI. Furthermore, the rA1 domain binds to prothrombin and A1-derived synthetic peptides inhibit this interaction (Figs. 3 and 4, Table I). In order to extend and confirm these observations, we have determined that both PF1.2 and PF2 bind FXI in a specific and saturable manner (Fig. 6, A and B). Data presented in Fig. 7A and Table I demonstrate that the two peptides, Phe56–Ser86 and Ala45–Arg54, also inhibit the binding of PF1.2 to FXI with IC50 values of 5 × 10^{-5} M and 6 × 10^{-5} M, respectively. Thus, these experiments confirm the conclusion from binding studies with immobilized prothrombin and FXI (above) that kringle II can bind to amino acids comprising the thrombin-binding site (Ala45–Arg54) and the HK-binding site (Phe56–Ser86) within FXI.

DISCUSSION

Previously, we identified a sequence of amino acids in FXI that binds thrombin and is juxtaposed to the HK binding site (15). We also investigated the role of PF1 and PF1.2 and prothrombin in their interaction with FXI since prothrombin is the precursor of thrombin (14). We recently reported that prothrombin as well as thrombin binds FXI at or near the HK binding site in the A1 domain (Phe56–Ser86) (14). These studies suggest that the kringle II domain of prothrombin binds at or near the HK binding site in the A1 domain (14). Additionally, PF1.2 abrogates the inhibitory effect of HK on thrombin-catalyzed FXI activation in the presence of dextran sulfate or activated platelets, indicating that it cannot bind to the same site in FXI as thrombin (14). To understand the physiological importance of the FXI-prothrombin interaction, we have attempted to identify the amino acid sequences in FXI that interact with prothrombin.

Our experiments support the conclusion that a sequence of amino acids (Ala45–Ser86) in the A1 domain of FXI that contains three antiparallel β-strands connected by β-turns comprises a surface that interacts with the kringle II domain of prothrombin. The evidence supporting this conclusion is as follows. 1) The rA1 domain inhibits the binding of prothrombin to FXI with an IC50 of 4 × 10^{-7} M (Fig. 1A, Table I). 2) According to surface plasmon resonance studies, prothrombin interacts with FXI (Ka(app) = 71.5 ± 14 nM) and with the rA1 domain of FXI (Ka(app) = 239 ± 83 nM) (Fig. 2 and Table II). 3) PF1.2 inhibits the binding of prothrombin to FXI (with a IC50 of 5 × 10^{-7} M) whereas PF1 has no effect (Fig. 1B, Table I). 4) The rA1 domain (Glu1–Ser90) binds prothrombin in a saturable manner with a Kd(app) value of 471 ± 175 nM (Fig. 3). 5) PF2 or PF1.2 binds FXI in a saturable manner with Ka(app) values of 417 ± 190 and 449 ± 172 nM, respectively (Fig. 6A and B). 6) Based on a molecular model of the A1 domain, conformationally constrained peptides were synthesized, which act synergistically to inhibit rA1 binding to prothrombin (Fig. 4, A and B) or PF2 binding to FXI (Fig. 6A and B, Table I). We conclude from these studies that the binding of prothrombin to FXI is mediated by amino acid residues (Ala45–Ser86) exposed on the surface of the A1 domain (Glu1–Ser90) that interact with complementary residues within the kringle II domain of prothrombin.

Our subsequent studies were focused on the identification and mapping of the site within FXI that mediates the binding of prothrombin employing a variety of solid phase binding assays and a kinetic method (utilizing surface plasmon resonance) in which prothrombin was bound to a carboxymethyl dextran (CM5) flow cell using amine coupling chemistry, for examining FXI binding to prothrombin. Although these two methods gave similar estimates for the affinity of FXI binding have determined that the binding of FXI to HK is mediated at least in part by Val64 and Ile77 in the A1 domain of FXI (29). Therefore, we examined the effects of mutations of these two residues on the capacity of the rA1 domain to inhibit rA1 binding to prothrombin. We found that the mutant rA1 domain constructs (V64A and I77A), which have lost the capacity to inhibit FXI binding to HK (29), retain the full capacity of the rA1 domain (Glu1–Ser90) to inhibit rA1 binding to prothrombin (Fig. 5C, Table I). Therefore, the binding sites for HK and prothrombin, although contiguous, are apparently separate and distinct.

The Binding of Prothrombin Fragment 1.2 or Prothrombin Fragment 2 to Factor XI—We have reported that PF1.2 inhibits the binding of FXI to HK, whereas PF1 has no effect (14). Our experiments suggest that prothrombin binds to FXI at a site spatially contiguous with the HK binding site and that PF1.2 (presumably using the kringle II domain) may also bind FXI near this site displacing HK from the A1 domain. In order to test this, we determined whether PF1.2 or PF2 binds directly to FXI bound to the wells of a microtiter plate. Our results indicate that 125I-PF2 binds FXI in a saturable manner with a Ka(app) of 417 ± 190 nM (Fig. 6A), while 125I-PF1.2 binds FXI in a saturable manner with a Ka(app) of 449 ± 172 nM (Fig. 6B).

The Effect of Apple 1-derived Peptides on the Binding of Prothrombin to FXI—Fig. 2. Binding of factor XI (A) and recombinant Apple 1 (B) to prothrombin detected by surface plasmon resonance. Prothrombin was immobilized either by amine coupling or aldehyde linkage of carbohydrate chains to a CM5 carboxymethyl dextran sensor chip. A, factor XI, at concentrations of 12.5, 25, 50, and 100 nm, was injected at a flow rate of 30 μl/min with 7-min association and dissociation times. B, recombinant Apple 1 domain was injected at concentrations of 125 nm, 250 nm, 500 nm, and 1 μM with 7 min association and dissociation times. The increase in response units at the beginning of the dissociation phase is an artifact resulting from closure of the injection port and opening of the port for buffer flow. Representative sensorgrams of three determinations at the same concentrations are shown.
to prothrombin ($K_d^{(app)} \approx 250 \text{ nM}$ utilizing the microtiter plate assay (Table I); $K_d^{(app)} \approx 70 \text{ nM}$ with the SPR method (Table II)), the latter kinetic method gives a more reliable estimate of affinity since the microtiter plate assay is likely to represent non-equilibrium binding measurements of a semiquantitative nature. Nonetheless, the microtiter plate assays yielded internally consistent results (comparing assays with either FXI or prothrombin immobilized and prothrombin, PF2, FXI, or rA1 domain as ligand) and provided a useful method for characterizing the prothrombin-kirngle II domain binding site in the FXI A1 domain. Moreover, the $K_d^{(app)} (250 \pm 50 \text{ nM})$ in the equilibrium binding experiments were almost identical to the $K_d^{(app)}$ in the surface plasmon resonance experiments in which prothrombin was bound to the rA1 (Glu1–Ser90) (239 ± 83 nm). Therefore, we conclude that prothrombin binds FXI entirely through the A1 domain. Furthermore, we conclude that FXI binds in a saturable and reversible manner to immobilized prothrombin and that the fidelity of FXI as a probe for quantitative assessment of this interaction is not affected by the radiolabeling procedure.

In order to identify specific amino acid residues within the A1 domain of FXI involved in binding either HK or thrombin, conformationally constrained peptides and rA1 domains were synthesized containing conservative amino acid substitutions suspected on the basis of molecular modeling of the A1 domain to contain side chains involved in these interactions (15, 29). Since the prothrombin binding site was localized to the A1 domain and found to contain amino acid sequences overlapping the thrombin and HK binding sites, we aimed to determine whether specific amino acid residues in the A1 domain involved in binding HK or thrombin might also bind prothrombin or kringle II (Figs. 4 and 6, Table I; Fig. 3).

**TABLE I**

| Protein            | Association rate constant | Dissociation rate constant | Calculated $K_d$ |
|--------------------|---------------------------|----------------------------|-----------------|
| Factor XI          | $8.36 \pm 0.39 \times 10^4$ | $4.51 \pm 0.41 \times 10^{-3}$ | $53.9 \pm 14$   |
| Recombinant Apple 1| $1.98 \pm 0.185 \times 10^4$ | $5.035 \pm 0.64 \times 10^{-3}$ | $254 \pm 83$    |

**FIG. 3.** Saturable binding of $^{125}$I-recombinant Apple 1 (Glu1–Ser90) to prothrombin. Prothrombin bound to microtiter plates was incubated with $^{125}$I-labeled rA1 (Glu1–Ser90) at various concentrations (50–3,000 nM). Amount of rA1 bound, expressed as a percent of total cpm bound after subtracting 220 cpm representing the negative control in which $^{125}$I-rA1 was added to wells coated with bovine serum albumin instead of prothrombin.

**FIG. 4.** Binding of $^{125}$I-recombinant Apple 1 (Glu1–Ser90) to prothrombin in the presence of various concentrations of Apple 1-derived peptides. $^{125}$I-rA1 (1,000 nM) was incubated with peptides or rA1 at the concentrations indicated. When prothrombin was not bound to the wells of microtiter plates, the amount of $^{125}$I-rA1 was <0.1% of the control value and the maximum variation of cpm bound for each experimental observation was <2% of total cpm bound. Zero A1 (Glu1–Ser90) bound (nM) represents the amount bound after subtracting 220 cpm representing a negative control in which $^{125}$I-rA1 was added to wells coated with bovine serum albumin instead of prothrombin.

31959

Prothrombin Kringle II Domain Binds to FXI Apple 1 Domain

in binding HK or thrombin might also bind prothrombin or kringle II. Our results are consistent with the following conclusions. 1) Val164 and Ile77, which are important as contact sites for HK (but not for thrombin), do not participate in the interaction of FXI with prothrombin or kringle II (Figs. 4 and 6,
Table I). 2) Glu66 and Asp51 which are important contact sites for the interaction of the A1 domain with thrombin (but not for HK), are not involved in the interaction of the A1 domain with prothrombin or kringle II (Fig. 5A, Table I).

We have utilized four different, complementary assays to investigate prothrombin interaction with FXI (Table I): 1) prothrombin binding to FXI, 2) PF1.2 binding to FXI, 3) rA1 binding to prothrombin, and 4) PF2 binding to FXI. We have previously reported that prothrombin binds FXI in a saturable, specific manner with a $K_d$ (app) of 250 nM (14) and now demonstrate that PF1.2 binds FXI in a saturable manner with a $K_d$ (app) of 449 ± 6172 nM (Fig. 6A). In order to examine direct interactions between the kringle II domain of prothrombin and FXI A1 domain, we devised two additional assays: PF2 binding to FXI and rA1 binding to prothrombin; both binding assays reveal essentially identical $K_d$ (app) values of 417 ± 190 nM and 471 ± 175 nM (Figs. 3 and 6A). The close correspondence of estimated affinities using these four different binding assays provides a basis for confidence in the validity of the results and the assumption that the conformation of FXI or prothrombin was not altered significantly when the proteins were bound to the wells of microtiter plates.

The plasma protein, PK, which shares a high degree of sequence identity (58%) with FXI, was also examined to determine whether homologous amino acid sequences can inhibit rA1 domain binding to prothrombin. Unlike the FXI peptides, the PK peptides, Pro45–Lys54 and Phe56–Gly86, did not inhibit rA1 binding to prothrombin (Fig. 4A, Table I). Therefore, PK, which binds HK in the Phe 56–Gly86 region (31) but does not bind thrombin (Pro 45–Lys54) (15), also does not compete with thrombin binding to FXI, 2) PF1.2 binding to FXI, 3) rA1 binding to prothrombin, and 4) PF2 binding to FXI. We have previously reported that prothrombin binds FXI in a saturable, specific manner with a $K_d$ (app) of 250 nM (14) and now demonstrate that PF1.2 binds FXI in a saturable manner with a $K_d$ (app) of 449 ± 6172 nM (Fig. 6B). In order to examine direct interactions between the kringle II domain of prothrombin and FXI A1 domain, we devised two additional assays: PF2 binding to FXI and rA1 binding to prothrombin; both binding assays reveal essentially identical $K_d$ (app) values of 417 ± 190 nM and 471 ± 175 nM (Figs. 3 and 6A). The close correspondence of estimated affinities using these four different binding assays provides a basis for confidence in the validity of the results and the assumption that the conformation of FXI or prothrombin was not altered significantly when the proteins were bound to the wells of microtiter plates.

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Prothrombin Kringle II Domain Binds to FXI Apple 1 Domain

FXI for binding sites on prothrombin.

In order to investigate the three stem-loop structures defined by the molecular model, the three peptides had cysteine residues introduced at the amino terminus and the carboxyl terminus of each peptide so that the resulting disulfide bond might stabilize the looplike structure (15, 18, 29). The individual loop-structures were tested in assays of rA1 binding to FXI, Ala45–Arg54 (●) on the binding of 125I-FF1.2 to FXI. B results represent the effects of FXI Phe56–Ser86 (○) and FXI Ala54–Arg64 (●) on the binding of 125I-FF1.2 to FXI. B, results represent the effects of FXI Phe56–Val71 (○), FXI Asn72–Ser86 (●), FXI Ala54–Arg64 (○), FXI Ala54–Arg64 + Phe56–Val71 at equimolar concentrations (●), FXI Ala54–Arg64 + Asn72–Ser86 at equimolar concentrations (●), FXI Phe56–Val71 + Asn72–Ser86 at equimolar concentrations (●), and FXI Ala54–Arg64 + Phe56–Val71 + Asn72–Ser86 at equimolar concentrations (●) on the binding of 125I-FF1.2 to FXI.

FIG. 7. The effect of factor XI Apple 1-derived peptides on the binding of 125I-prothrombin fragment 1.2 (A) and 125I-prothrombin fragment 2 (B) to factor XI. 125I-PPF1.2 (722 nM) or 125I-PPF2 (820 nM) were incubated with the various peptides at the concentrations indicated. When FXI was not bound to the wells of the microtiter plates, the amount of 125I-PPF1.2 or 125I-PPF2 was <0.2% of the control value and to maximum variation of cpm bound for each experimental observation was <2% of total cpm bound. One hundred percent binding of PPF1.2 or PPF2 represents an average of 19,200–20,164 cpm bound, whereas 0% binding of FXI represents the amount bound after subtracting 110 and 105 cpm representing the negative control in which 125I-PPF1.2 or 125I-PPF2 was added to wells coated with bovine serum albumin instead of FXI. A, results represent the effects of FXI Phe56–Ser86 (○) and FXI Ala54–Arg64 (●) on the binding of 125I-FF1.2 to FXI. B, results of FXI Phe56–Val71 (○), FXI Asn72–Ser86 (●), FXI Ala54–Arg64 (○), FXI Ala54–Arg64 + Phe56–Val71 at equimolar concentrations (●), FXI Ala54–Arg64 + Asn72–Ser86 at equimolar concentrations (●), FXI Phe56–Val71 + Asn72–Ser86 at equimolar concentrations (●), and FXI Ala54–Arg64 + Phe56–Val71 + Asn72–Ser86 at equimolar concentrations (●).

main to prothrombin with significantly greater potency than any of the stem loop peptides individually indicating synergistic interactions between the three conformationally constrained loop structures in comprising a binding site within the FXI A1 domain for the kringle II domain of prothrombin.

Since prothrombin (and PF1.2 but not PF1) binds to FXI through the A1 domain and displaces HK, it obviates the inhibitory effect of HK on thrombin-catalyzed F-XI activation on the platelet surfaces (14). We were therefore led to postulate that complex formation between FXI and prothrombin promotes the binding of FXI to the platelet surface (14). Our studies demonstrate that prothrombin (in the presence of Ca2+ ions) can substitute for HK (and Zn2+) as a cofactor for FXI binding to activated platelets (14). Therefore, it was important to examine the interaction of FXI and prothrombin. Our results suggest that prothrombin contains two separate and distinct binding sites, one in the kringle II domain of prothrombin, the other in the catalytic domain of thrombin, both of which bind to the A1 domain of FXI thereby displacing HK without displacing each other (14). We have mapped the thrombin binding site to spanning residues Ala45–Arg54 (15) and the HK binding site to residues Phe56–Ser86 (8, 29), whereas the prothrombin (kringle II) binding site was found in the present study to reside within amino acid residues Ala54–Ser86. Thus, the data in this paper and previously published studies support the conclusion that prothrombin (kringle II), thrombin, and HK binding sites are all contained within the A1 of FXI and, although contiguous, are separate and distinct. However, these three binding sites overlap since they share a common sequence of amino acids. Our results are consistent with the conclusion that prothrombin binding to the A1 domain facilitates FXI binding to the activated platelet surface, thereby favoring thrombin-mediated activation of FXI (14). FXI binding to prothrombin is also predicted to block HK binding and to prevent FXIIa-catalyzed activation of FXI. Evidence to confirm this prediction has recently been reported (32). We have suggested that the interaction of FXI with prothrombin results in a conformational transition in FXI resulting in the exposure of a binding site within the A3 domain (Asn235–Arg266) of FXI that is important in mediating a direct, high affinity interaction with the platelet surface (14). Therefore, platelet-bound FXI can be activated efficiently by thrombin (14). The presence of prothrombin at physiological concentration would favor this mechanism supporting the hypothesis that thrombin can activate the intrinsic pathway in a revised model of platelet-dependent blood coagulation (8, 9, 14).

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