The Role of High Molecular Weight Kininogen and Prothrombin as Cofactors in the Binding of Factor XI A3 Domain to the Platelet Surface*

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We have reported that prothrombin (1 μM) is able to replace high molecular weight kininogen (45 nM) as a cofactor for the specific binding of factor XI to the platelet (Baglia, F. A., and Walsh, P. N. (1998) Biochemistry 37, 2271–2281). We have also determined that prothrombin fragment 2 binds to the A1 domain of factor XI at or near the site where high molecular weight kininogen binds. A region of 31 amino acids derived from high molecular weight kininogen (HK31-mer) can also bind to factor XI (Tait, J. F., and Fujikawa, K. (1987) J. Biol. Chem. 262, 11651–11656). We therefore investigated the role of prothrombin fragment 2 and HK31-mer as cofactors in the binding of factor XI to activated platelets. Our experiments demonstrated that prothrombin fragment 2 (1 μM) or the HK31-mer (8 μM) are able to replace high molecular weight kininogen (45 nM) or prothrombin (1 μM) as cofactors for the binding of factor XI to the platelet. To localize the platelet binding site on factor XI, we used mutant full-length recombinant factor XI molecules in which the platelet binding site in the Apple 3 domain was altered by alanine scanning mutagenesis. The recombinant factor XI with alanine substitutions at positions Ser248, Arg250, Lys255, Leu257, Phe260, or Gln263 were defective in their ability to bind to activated platelets. Thus, the interaction of factor XI with platelets is mediated by the amino acid residues Ser248, Arg250, Lys255, Leu257, Phe260, and Gln263 within the Apple 3 domain.

Coagulation factor XI (FXI) is a disulfide-linked homodimeric protein (160,000 Da) (1) that is cleaved by thrombin, FXIIa, or FXIa at a single peptide bond (Arg269–Ile270) to give rise to FXIa (2–5). The primary structures of four repeat sequences (designated A1, A2, A3, and A4 or Apple domains) present in the heavy chain region of FXI have been elucidated by the 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 1754 solely to indicate this fact.

To whom correspondence should be addressed: Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. Bacto-tryptone, Bacto-peptone, ligase, dNTPs, and Sephadex G-50 (fine) were purchased from Amersham Pharmacia Biotech. The human FXI cDNA was a gift from Dr. Werner Muller-Esterl (University of Mainz, Mainz, Germany). The Chameleon site-directed mutagenesis kit and plasmid pBluescript (SK+ version) were purchased from Stratagene (La Jolla, CA). The 293 human fetal kidney fibroblast cell line was purchased from the American Tissue Type Collection (Manassas, VA). Bacto-tryptone, Bacto-

EXPERIMENTAL PROCEDURES

Materials—Human FXI and FXIa were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). DNA markers, Pfu polymerase, ligase, dNTPs, and Sphadex G-50 (fine) were purchased from Amersham Pharmacia Biotech. The human FXI cDNA was a gift from Dr. Dominic Chung (University of Washington, Seattle, WA). The region of 31 amino acids derived from the HK31-mer was a gift from the Chameleon site-directed mutagenesis kit and plasmid pBluescript (SK+ version) were purchased from Stratagene (La Jolla, CA). The 293 human fetal kidney fibroblast cell line was purchased from the American Tissue Type Collection (Manassas, VA). Bacto-tryptone, Bacto-

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yeast extract, Dulbecco's modified Eagle medium, and G418 were obtained from Life Technologies, Inc. Cellgro Complete serum-free medium was purchased from Mediatech (Herndon, VA). Trichloroacetic acid, β-mercaptoethanol, and disodium EDTA were purchased from Fisher Scientific (Springfield, NJ). Methyl silicon oil (1 DC-200) and Hi phospholipid (1 DC-550) were purchased from William F. Nye, Inc. (Fairhaven, MA). Carrier free Na125I was purchased from Ameri- sham Pharmacia Biotech. Goat anti-human FXI polyclonal IgG, with and without conjugated horseradish peroxidase, for FXI enzyme-linked immunosorbent assay were from Enzyme Research Laboratories (South Bend, IN). Alkaline phosphatase-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch Laboratory Inc. (West Grove, PA). The chromogenic substrate for measurement of FXI activity (S2366) was obtained from Chromogenix (Molndal, Sweden). Rabbit brain phospholipids (Cephaline) were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). The thrombin receptor agonist peptide, SFLLRN-amide, was synthesized at the Protein Chemistry Facility of the University of Pennsylvania on a 430A synthesizer (Applied Biosystems, Foster City, CA) and purified by reverse-phase high performance liquid chromatography to >99% homogeneity. Normal pooled and FXI-deficient human plasmas were purchased from George King Biochemical (Overland Park, KS). All reagents and materials used for SDS-polyacrylamide gel electrophoresis were purchased from Bio- Rad. IODOGEN vials (20 μg of coating) were obtained from Pierce. Peroxidase-conjugated goat anti-rabbit, lima bean trypsin inhibitor, aprotinin, benzamidine, HEPES, sodium periodate, Sepharose 2B-CL, Sephadex G25 (fine), dithiothreitol, sodium phosphate, Tris-HCl, Trizma base, cytoine, bovine serum albumin, iodoacetamide, and metabisulfite were purchased from Sigma.

**Radiolabeling of Proteins—**Purified proteins were radiolabeled by a minor modification of the IODOGEN method. Briefly, 2 μl of Na125I (500–1,000 μCi/μl) was added to 100 μl of protein (0.1–1.0 mg/ml) and incubated in an IODOGEN vial (20 μg coating) for 20 min. The reaction was stopped by adding metabisulfite (50 μg/ml). The 125I-protein was purified by centrifugation at 200 × g for 20 min through a 1.0-ml Sepha- dex G-50 column equilibrated in 0.1 M Tris pH 8.0. The incorporation of 125I into FXI was >98%, and the specific radioactivity of the 125I-FXI was 29.4 ± 0.4 μCi/μg. This corresponds to 1.24 ± 0.40 atoms of 125I/molecule of FXI monomer.

**Preparation of Wild Type and Mutant Human FXI**—The procedures utilized to prepare wild type FXI and the FXI mutants rFXI(S248A), rFXI(T249A), rFXI(R250A), rFXI(I251A), rFXI(K252A), rFXI(K253A), rFXI(249–251 A), rFXI(F260A), rFXI(Q263A), rFXI(S254A), rFXI(K255A), rFXI(L257A), rFXI(257–260 A), rFXI(S258A) and several others were introduced into the wild type human FXI cDNA in vector pBluescript (SK+) version) using a Chameleon site-directed mutagene- sis kit, according to the manufacturer’s recommendations. Proper intro- duction of the mutation was confirmed by conventional dideoxy chain termination DNA sequencing. cDNAs were subsequently ligated into a mammalian expression vector, pJVC-MV, containing the cytomegalo- virus promoter. Human fetal kidney fibroblasts (293 cells, 5 × 10^6 cells) were co-transfected with 40 μg of FXI cDNA/pJVC-MV construct and 2 μl of a plasmid RSVneo, which contains a gene conferring resistance to neomycin. Transfection was by electroporation using an Electrozell Manipulator 600 (BTX, San Diego, CA). Transfected cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum for 24 h and then switched to the same medium supplemented with the neomycin analog, G418, at 500 μg/ml. Medium was exchanged every 48 h. G418-resistant clones were transferred to 24-well tissue culture plates and cultured to confluence in 175-cm2 tissue culture flasks. When the cells had reached confluence, medium was replaced with 75 ml of serum-free Cellgro complete medium supplemented with 10 μg/ml soybean trypsin inhibitor, 10 μg/ml lima bean trypsin inhibitor, and 5 μg/ml aprotinin. This mixture of protease inhibitors prevents activation or degradation of the recombi- nant protein in the conditioned medium. Medium was exchanged every 48 h. rFXI-Q263A were those described by Sun et al. (1993) purchased from Bio- Rad. IODOGEN vials (20 μg coating) were purchased from Bio- Rad. Carrier free Na125I was used to prepare 125I-labeled FXI by incubation with FXI for 20 min. 125I-FXI was removed to a new 50-ml tube and centrifuged again at 800 × g for 5 min to remove contaminating red blood cells. This supernatant was centrifuged at 1,800 × g for 15 min. The blood was resuspended in 5 ml of Hi phospholipid buffer, pH 6.5, and filtered on a column of Sepha- dex G-25 equilibrated in Hi phospholipid buffer, pH 7.4. Platelets were collected using a particle counter from Coulter Electronics (Hialeah, FL).

**Coagulation Assays—** The coagulation assays were performed using the kaolin-activated partial thromboplastin time. The master reaction mixture contained 50 μl of 25 mg of kaolin mixed with phospholipids (0.04% inosine) in 5 ml of HEPECS-Tyrode buffer, pH 7.4. To this mixture, 50 μl of FXI-deficient plasma, 1 nm wild type FXI, or appro- priate concentrations of recombinant wild type FXI or mutant rFXI-A3 proteins were added to a final volume of 200 μl. This was repeated with activated platelets (100 μl) substituted for phospholipids. The master mixture was incubated at 37 °C for 5 min; 50 μl of 50 mCi CaCl2 was then added, and the clotting time was determined.

**Isolation and Purification of Prothrombin Fragment 2 (PF2)** (Se206 to Arg212) from Prothrombin—Prothrombin activation material containing PF2 and depleted of thrombin by SP-Sephadex column chromatog- raphy was obtained from Enzyme Research Laboratories (South Bend, IN) and processed as follows. This preparation contained 0.4 mg/ml protein in 50 mM sodium phosphate and 1 μM benzamidine, pH 6.5. To 50 ml of this material, Ph-Pro-Arg-chloromethyl ketone (Calbiochem, Indianapolis, IN) was added to a concentration of 10 μM. PF2 was isolated using fast protein liquid chromatography at 23 °C using a sephadex column, according to the procedure of Su and Wren (1995) or the procedure of Su and Wren (1995). Preparative isolation of the material on this column results in PF1 and PF2 in two separate pools. PF2 migrates at 14 kDa as a doublet on SDS-gel electrophoresis. From 50 ml loaded on the column, approximately 7 mg of PF2 was recovered. An identical prepara- tion of human PF2 was obtained as a generous gift from S. Krish- nawamy (University of Pennsylvania, Philadelphia, PA) and gave identical results. Pf2 was dialyzed against Tris-buffered saline, and stored at 4°C until use.

**Platelet Binding Experiments—** Direct binding and competition ex- periments were carried out as described previously (16). For direct binding, platelets (10^9/ml) were activated with the thrombin receptor activation peptide (5 μM, SFLLRN-amide) for 5 min at 37 °C. Then, various concentrations of 125I-FXI and the following reagents were added in the combinations specified in the figure legends: 25 μM ZnCl2, and/or 2 mM CaCl2, with 50 mM HK, 8 μM HK31-mer, 1 μM prothrombin, or 1 μM PF2. For total binding, unlabelled FXI was not added in the reaction mix; however, for determination of nonspecific binding, a 50-fold molar excess of unlabelled FXI was added in the reaction mix. At the end of the incubation period (30 min, 37 °C), 100-μl aliquots were removed and centrifuged through a mixture of Dow Corning methyl silicon oil (3 parts of 550 density oil for every 2 parts of 200 density oil) to separate platelet pellets from supernatant. The amount of 125I-FXI bound to platelets was counted for 125I using a Wallac 1470 Wizard γ counter. Specific binding was determined as the difference between total binding and nonspecific binding.

In competition experiments, the concentration of competitor that displaced 50% of bound 125I-FXI (IC50) was determined by plotting the 125I-FXI bound to platelets versus the concentration of competitor ligand added. The Ki was calculated using the equation: IC50 = (1 + [S]/Ki) · Kc, where Kc was the concentration of 125I-FXI used in these experiments (held constant at 22 nM) and IC50 was the value (10 nM) determined from direct binding experiments. One hundred percent binding of 125I-FXI represented an average of ~550,000 cpm bound whereas 0% binding represented background binding (~250 cpm).
Binding of Factor XI and Factor XI Mutants to High Molecular Weight Kininogen on Surface Plasmon Resonance—Binding studies were performed on a Biacore 2000 flow biosensor (Biacore, Inc., Uppsala, Sweden). High molecular weight kininogen was immobilized on a carboxymethyl dextran (CM5) flow cell surface using amine coupling chemistry. Briefly, 1.15 mg of N-hydroxysuccinimide was mixed with 7.5 mg of N-(3-dimethylamino)propylcarbodiimide hydrochloride and injected at 5 μl/min, for 7 min across the flow cell surface. HK, ~10 μg/ml in sodium acetate buffer, pH 4.5, was then injected for 1–2 min to a response level of ~500 response units. Any remaining derivatized carboxymethyl groups were then blocked by a 7-min injection of 1 M N-hydroxysuccinimide. Serial dilutions of wild type FXI and FXI mutants in HBSS, with or without 20 μM zinc, 0.005% surfactant P20 (Biacore, Inc.) were injected with a 6-min association time and 5-min dissociation time. After subtraction of the nonspecific binding curves, the association and dissociation rate constants were determined using a global fit to a one to one Langmuir association model on Biacore evaluation 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 for the highest concentration. The χ² value is the square of the differences between the theoretical ideal curve and the actual curve and was calculated according to the following equation.

\[
\chi^2 = \frac{\sum (r_i - r_0)^2}{n - p}
\]  

(1)

Where \( r_i \) indicates the fitted value at a given point, \( r_0 \) indicates the experimental value at that point, \( n \) indicates the number of data points, and \( p \) indicates the number of fitted parameters.

Statistics—Results are expressed as the mean ± S.E. Statistical analysis was performed with Student’s t test. \( p < 0.05 \) was considered to be statistically significant.

RESULTS

The Effect of HK31-mer (Ser^194 to Lys^224) and Prothrombin Fragment 2 (PF2) (Ser^156 to Arg^271) on the Binding of Factor XI to the Activated Platelet—We reported that prothrombin binds to the Activated Platelet—

- In the absence of zinc, the saturable binding decreases by 70%, as compared with total binding in the presence of zinc (Fig. 1). However, when we replaced zinc with calcium, we observed suboptimal binding (Fig. 1), suggesting that in addition to the HK31-mer, the binding of FXI to activated platelets also requires zinc ions.

- Furthermore, when compared with prothrombin and HK as cofactors for FXI binding to activated platelets, the HK31-mer and PF2 peptides were able to induce saturable binding in the same manner as the intact proteins (Fig. 1). The results demonstrate that saturable binding was achieved at FXI concentrations above those present in plasma (30 nm). When saturation binding data were analyzed, the \( K_d \) (10 nm) and total number of sites (1,500–2,000 sites/platelet) were the same for HK, HK31-mer, prothrombin, and PF2. Thus, the synthetic peptide HK31-mer or PF2 can substitute for HK or prothrombin, respectively, as a cofactor for FXI binding to platelets.

- We also examined the binding of FXI to activated platelets in the presence or absence of ions. When HK31-mer was used as a cofactor, in the absence of zinc, the saturable binding decreased by 70%, as compared with total binding in the presence of zinc (Fig. 1). However, when we replaced zinc with calcium, we observed suboptimal binding (Fig. 1), suggesting that in addition to the HK31-mer, the binding of FXI to activated platelets also requires zinc ions.
Factor XI Does Not Use High Molecular Weight Kininogen or Prothrombin as a Receptor for Binding to Activated Platelets—The ability of cofactors to act as co-receptors for FXI binding to activated platelets was examined. The binding of 125I-FXI (50 nM) in the presence of either HK (50 nM), Ca\(^{2+}\) (2 mM), Zn\(^{2+}\) (25 \(\mu M\)), and various concentrations of HK31-mer (A) or prothrombin (1 \(\mu M\), Ca\(^{2+}\) (2 mM), Zn\(^{2+}\) (25 \(\mu M\)), and various concentrations of PF2 (B). Experimental details are provided under “Experimental Procedures.”

When PF2 was used as cofactor, the binding was specific and saturable in the presence of zinc. In the absence of zinc, specific binding was reduced by 75%. When calcium was included in the binding assay, slight enhancement of binding was observed (Fig. 1), suggesting that the requisite ion for PF2-mediated binding of FXI is zinc and not calcium as previously reported for prothrombin (15).

Factor XI Mutants to High Molecular Weight Kininogen—To assure that replacement of the A3 domain of FXI with that of PK did not affect the capacity of FXI to bind to HK, binding studies were carried out using surface plasmon resonance. Additionally, either recombinant wild type FXI or FXI mutants with single alanine substitutions at selected residues within the A3 domain of FXI (S248A, R250A, K252A, K253A, K255A, L257A, F260A, and Q263A), or triple alanine mutants (249–251 \(\rightarrow\) A), (252–254 \(\rightarrow\) A), (255–257 \(\rightarrow\) A), were examined for their ability to bind to activated platelets via HK- or prothrombin-binding sites on platelets, then high concentrations of HK31-mer or PF2 would prevent FXI binding. The results indicated that the binding of FXI to platelets remained unchanged at high concentration of HK31-mer or PF2. Thus, these cofactors do not function as receptors for FXI on the platelet surface. This study also suggests that, even if the HK31-mer or PF2 are displacing HK or prothrombin from FXI, the platelet binding site on the A3 domain of FXI remains exposed and functional due to continued occupancy of the HK or prothrombin binding site in the FXI A1 domain by the HK31-mer or by PF2.

Binding of Factor XI to Platelets Is Mediated through the A3 Domain of FXI—We have reported that a platelet-binding site exists in the A3 domain of FXI. In order to expose this site, FXI must be bound to a cofactor (HK or prothrombin) (10, 16) or alternatively, as shown in Fig. 1, to PF2 or to the HK31-mer peptide.

Prekallikrein (PK), another blood coagulation factor with four tandem repeats that are 58% identical to FXI (17, 26), was examined to determine if it contains a platelet binding site. Our experiments demonstrated that neither PK nor a chimeric protein in which the A3 domain of FXI was replaced by that of PK (FXI-PKA3) binds saturably or specifically to the activated platelet surface (Fig 3) in the presence of HK. This result demonstrates that the A3 domain of FXI mediates its binding to activated platelets. Since this FXI-PKA3 protein binds normally to HK (see next section, below) this result also supports the conclusion that HK does not comprise a binding site for FXI on the platelet surface.

### Table I

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<th>(k_{on}) (\times 10^8 M^{-1} s^{-1})</th>
<th>(k_{off}) (s^{-1})</th>
<th>(K_d) (nM)</th>
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### Fig. 2

Effects of HK31-mer peptide or PF2 on binding of FXI to activated platelets in the presence of HK or prothrombin. Radiolabeled FXI (50 nM) was incubated with activated platelets (10\(^8\)/ml) in the presence of either HK (50 nM), Ca\(^{2+}\) (2 mM), Zn\(^{2+}\) (25 \(\mu M\)), and various concentrations of HK31-mer (A) or prothrombin (1 \(\mu M\), Ca\(^{2+}\) (2 mM), Zn\(^{2+}\) (25 \(\mu M\)), and various concentrations of PF2 (B). Experimental details are provided under “Experimental Procedures.”

### Fig. 3

Binding of FXI to platelets is mediated through the A3 domain of FXI. Direct binding experiments were conducted examining the binding of 125I-FXI (○), 125I-PK (□), or 125I-rFXI-PKA3 (△) to activated platelets (\(\sim\)10\(^9\)/ml) using 50 \(\mu M\) HK and 25 \(\mu M\) Zn\(^{2+}\) as cofactors. Experiment conditions were as specified under “Experimental Procedures.” Data shown are mean values ± S.E. of specific binding results in three separate experiments, each carried out in triplicate.
Determination of Apple 3 Domain Residues That Comprise the Platelet Binding Site in Recombinant Factor XI.—Competition binding studies were carried out with rFXI, in which specific amino acid substitutions were made (alanine multiple and single scanning), to provide clues concerning the residues that may be important for binding to platelets in the A3 domain of FXI (Fig. 4, Table II). Substitutions with alanine at Ser248, Arg250, Lys252, Phe260, and Gln263 disrupted the ability of rFXI to compete normally with 125I-FXI binding to activated platelets ($K_i \approx 5 \times 10^{-8} \text{ M}$, $6 \times 10^{-7} \text{ M}$, $3 \times 10^{-7} \text{ M}$, $1 \times 10^{-7} \text{ M}$, and $5 \times 10^{-6} \text{ M}$, respectively). In contrast, none of the FXI mutants T249A, I251A, K252A, K253A, S254A, and S264A (all $K_i \approx 1 \times 10^{-8} \text{ M}$) showed a defect in platelet binding (Table II). Two of the multiple alanine scanning substitutions showed quantitatively similar defects, i.e. 250–255→A ($K_i \approx 9 \times 10^{-6} \text{ M}$), and 249–251→A ($K_i \approx 3 \times 10^{-6} \text{ M}$), whereas 252–254→A showed no deficiency ($K_i \approx 1 \times 10^{-8} \text{ M}$) in its ability to compete with 125I-FXI for binding to platelets.

The Effects of Mutant Recombinant Factor XI Proteins in Coagulant Assays Containing Platelets or Phospholipids.—A previous report from our laboratory showed that the A3 domain of FXI inhibited procoagulant activity in the presence of platelets but failed to inhibit procoagulant activity in the presence of phospholipids (17). We, therefore, carried out partial thromboplastin time coagulation assays to determine the effects of rFXI mutations on their abilities to promote surface-mediated coagulation. When phospholipids (0.04% inositol) were used as a surface, the procoagulant activities of all mutants were normal (Table III). However, when platelets were used as a surface (10^5 platelets/ml), the rFXI-PKA3 chimera had $\approx 0.5\%$ FXI activity. Additionally, the following mutants showed statistically significant ($p < 0.05$) defects in their FXI activity in the presence of platelets: S248A (67% FXI activity), R250A (20% FXI activity), K252A (40% FXI activity), L257A (50% FXI activity), F260A (8% FXI activity), Q263A (64% FXI activity), 250–255→A (5% FXI activity), 249–251→A (15% FXI activity), and 255–257→A (35% FXI activity). However, the following FXI mutants were shown, statistically, to be normal in activity: 125I-FXI (85% FXI activity), K252A (88% FXI activity), K253A (80% FXI activity), S254A (82% FXI activity), and S264A (78% FXI activity) (Table III). Thus, the mutants shown to be normal in binding to platelets were normal in their capacity to promote platelet-mediated FXI activity, whereas the mutants shown to be abnormal in platelet binding (S248A, R250A, K255A, L257A, F260A, and Q263A) were also abnormal in platelet-mediated FXI activity. Therefore, these experiments indicate that FXI interacts specifically with the platelet surface through the A3 domain and that FXI interaction with the platelet surface is an important physiological process.

DISCUSSION

We have previously demonstrated that both HK and prothrombin bind FXI with $K_a$ values of $10^{-8} \text{ M}$ and $K_d \approx 10^{-7} \text{ M}$, respectively (15). HK and prothrombin bind the A1 domain of FXI and can both act to promote the specific, high affinity binding of FXI to the surface of activated gel-filtered platelets (15, 16). Additionally, it is well documented that both HK (19) and prothrombin (18) bind specifically and with high affinity to the surface of activated platelets. It has previously been un-

FIG. 4. Fine mapping of the platelet binding site using rFXI mutants. Competition binding experiments were conducted in which various rFXI mutants were used as competitive ligands for 22 nM 125I-FXI binding to activated platelets in the presence of 50 nM HK and 25 μM Zn^2+. A, multiple alanine substitutions: rFXI(250–255→A); rFXI(250–255→A), where residues Arg250, Lys252, Lys255, and Lys265 were substituted with alanines, while residues 251 and 254 were the same as wild type FXI (•); rFXI(249–251→A), where all three residues were replaced with alanine (○); and rFXI(255–257→A), where all three residues were replaced with alanine (△). B, single alanine substitutions: rFXI(S248A) (●); rFXI(R250A) (▲); rFXI(K252A) (▼); rFXI(K255A) (▲); rFXI(K253A) (×); rFXI(F260A) (●); and rFXI(Q263A) (▲). C, results from single alanine substitutions that do not show any effect on 125I-FXI binding to platelets in the binding competition studies (T249A, I251A, K252A, K255A, and S264A). Control experiments were as specified under "Experimental Procedures." Data shown are mean values (± S.E.) of total binding results in three separate experiments, each carried out in triplicate.
clear whether HK and prothrombin promote the binding of FXI to the platelet surface by acting as a receptor for binding FXI to the platelet or whether alternatively they might bind to the FXI A1 domain, possibly to induce a conformational alteration in FXI resulting in the exposure of a platelet binding site in the A3 domain. In order to determine the mechanism by which these cofactors act, we compared the saturable binding to platelets of 125I-FXI in the presence of the known amino acid sequences of HK and prothrombin (HK31-mer and PF2) that interact with FXI and to the platelet surface (14, 15). Our present studies support the conclusion that prothrombin and HK bind to FXI and promote exposure of the platelet binding site in the A3 domain. The evidence supporting this possibility is as follows.

1) Saturation binding data (i.e., $K_d$ and number of sites on platelets) were approximately the same when HK or HK31-mer was used as a cofactor for FXI binding to the platelets (Fig. 1A). 2) Saturation binding data were approximately the same when prothrombin or PF2 was used as a cofactor for FXI binding to the platelets (Fig. 1B). 3) An increasing amount of cofactor (HK31-mer or PF2) did not decrease the amount of 125I-FXI binding to the platelet surface (Fig. 2). Thus, these cofactors are not receptors for FXI on the platelet surface.

The results of this study also confirmed our previous observation (15) that prothrombin can substitute for HK as a cofactor for the binding of FXI to activated platelets (Fig. 1). We previously reported that FXI binding to activated platelets in the presence of the cofactor HK requires the presence of $Zn^{2+}$ ions, whereas when prothrombin replaced HK as the cofactor, the divalent metal ion required was $Ca^{2+}$ (15). These metal ion requirements were rationalized as consistent with: 1) the presence of $Zn^{2+}$ binding site in HK (27) and the known $Zn^{2+}$ requirement for binding of HK to activated platelets (19), and 2) the requirement for calcium binding to the $\gamma$-carboxyglutamic acid domain of prothrombin, which is required for binding of prothrombin to activated platelets (28). However, when the peptides, HK31-mer or PF2, were used as cofactors to promote FXI binding to activated platelets, optimal binding ($K_d$ $\sim$ 10 nM, $\sim$1,500 sites/platelet) was observed in the presence of added $ZnCl_2$ (25 $\mu$M), whereas $CaCl_2$ (2 mM) was less effective (Fig. 1). Further studies will be required to facilitate a precise understanding of the divalent metal ion requirements and mechanism, especially since both calcium ions and zinc ions are stored in platelet-dense granules and are released in soluble form (29, 30) when platelet secretion is induced by agonists such as the thrombin receptor activation peptide (SFLLRN amide), which was employed for platelet activation in the present study.

It has been shown that both HK (19), through its heavy and light chains ($K_d$ $\sim$32 nM), and prothrombin (18), through its $\gamma$-carboxyglutamic acid domain ($K_d$ $\sim$320 nM, $\sim$15,000 sites/platelet), bind to activated platelets (15). We have previously reported that FXI binds to the surface of activated platelets ($\sim$1,500 sites/platelet) with a $K_d$ $\sim$20 nM (15, 16, 22, 25). We have also demonstrated that the A3 domain of FXI is directly involved in binding to platelets and can also compete with FXI for binding to the platelet surface (17). We have previously hypothesized that a higher affinity intermediate complex is formed, whereby FXI is initially bound to the platelet through sites exposed when FXI is bound to the kringle II domain of prothrombin or HK (15). However, the data presented in this paper suggest that intact HK or prothrombin are not required to generate this high affinity site for the platelet surface in the

### Table II

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK</td>
<td>5 ± 3 x 10^-9</td>
</tr>
<tr>
<td>PK (plasma-derived)</td>
<td>No binding*</td>
</tr>
<tr>
<td>rFXI - PKA3</td>
<td>No binding</td>
</tr>
<tr>
<td>rFXI(S248A)</td>
<td>5 ± 2 x 10^-6</td>
</tr>
<tr>
<td>rFXI(T249A)</td>
<td>1 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(R250A)</td>
<td>6 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(K251A)</td>
<td>1 ± 2 x 10^-6</td>
</tr>
<tr>
<td>rFXI(K252A)</td>
<td>1 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(K253A)</td>
<td>1 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(S254A)</td>
<td>1 ± 2 x 10^-6</td>
</tr>
<tr>
<td>rFXI(K255A)</td>
<td>3 ± 1 x 10^-7</td>
</tr>
<tr>
<td>rFXI(L257A)</td>
<td>1 ± 1 x 10^-7</td>
</tr>
<tr>
<td>rFXI(F260A)</td>
<td>5 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(Q263A)</td>
<td>2 ± 1 x 10^-7</td>
</tr>
<tr>
<td>rFXI(S264A)</td>
<td>1 ± 1 x 10^-8</td>
</tr>
<tr>
<td>rFXI(K250–255→A)*</td>
<td>No binding</td>
</tr>
<tr>
<td>rFXI(249–251→A)</td>
<td>3 ± 3 x 10^-6</td>
</tr>
<tr>
<td>rFXI(252–254→A)</td>
<td>1 ± 1 x 10^-6</td>
</tr>
<tr>
<td>rFXI(255–257→A)</td>
<td>6 ± 3 x 10^-7</td>
</tr>
</tbody>
</table>

* For the mutant rFXI(250–255→A), only the basic amino acids at positions 250, 252, 253, and 255 were changed to alanine. The amino acids at the positions 251 and 254 are the same as wild type FXI. No binding, no binding observed at the concentration tested.
A3 domain of FXI, provided the HK31-mer peptide or PF2 are present at concentrations required to saturate their A1 domain binding sites. Therefore, we have revised our model of the roles of prothrombin and HK in FXI binding to activated platelets as depicted in Fig. 5. Initially, FXI interacts with either HK or prothrombin in plasma, forming an intermediate complex that results in the exposure of the platelet binding site within the A3 domain. Upon platelet activation, the A3 domain mediates the direct, high affinity interaction between the FXI A3 domain and a receptor on the platelet surface.

Our previously published work (17) used recombinant A3 domains and synthetic peptides to fine map the platelet binding site on the A3 domain. In the present study, we utilized the rFXI molecule to confirm and refine our understanding of the role of the cofactors and the platelet binding site in binding FXI to the platelet surface. Our binding competition experiments demonstrate that prekallikrein and the rFXI-PKA3 chimera do not bind the activated platelet surface in the presence of HK and ZnCl₂ (Fig. 3), although they bind with normal affinity to HK (Table I).

Previously, using synthetic peptides and rA3 domain, we identified residues Arg²⁵⁰, Lys²⁵⁵, Phe²⁶⁰, and Gln²⁶³ as important for platelet binding (17). Single and multiple alanine scanning identified residues Arg²⁵⁰, Lys²⁵⁵, Phe²⁶⁰, and Gln²⁶³ as important for platelet binding (17). Initially, FXI interacts with either HK or prothrombin in plasma, forming an intermediate complex that results in the exposure of the platelet binding site within the A3 domain. Upon platelet activation, the A3 domain mediates the direct, high affinity interaction between the FXI A3 domain and a receptor on the platelet surface.

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The Role of High Molecular Weight Kininogen and Prothrombin as Cofactors in the Binding of Factor XI A3 Domain to the Platelet Surface
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