Factor XI Binding to Activated Platelets Is Mediated by Residues R^{250}, K^{255}, F^{260}, and Q^{263} within the Apple 3 Domain†

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ABSTRACT: To localize the platelet binding site on factor XI, rationally designed, conformationally constrained synthetic peptides were used to compete with [^{125}I]factor XI binding to activated platelets. The major platelet binding energy resided within the sequence of amino acids T^{249}−F^{260}. Homology scanning, using prekallikrein amino acid substitutions within the synthetic peptide T^{249}−F^{260}, identified a major role for R^{250} in platelet binding. Inhibition of [^{125}I]factor XI binding to activated platelets by the recombinant Apple 3 domain of factor XI and inhibition by unlabeled factor XI were identical, whereas the recombinant Apple 3 domain of prekallikrein had little effect. A "gain-of-function" chimera in which the C-terminal amino acid sequence of the Apple 3 domain of prekallikrein was replaced with that of factor XI was as effective as the recombinant Apple 3 domain of factor XI and unlabeled factor XI in inhibiting [^{125}I]factor XI binding to activated platelets. Alanine scanning mutagenic analysis of the recombinant Apple 3 domain of factor XI indicated that amino acids R^{250}, K^{255}, F^{260}, and Q^{263} (but not K^{252} or K^{253}) are important for platelet binding. Thus, the binding energy mediating the interaction of factor XI with platelets is contained within the C-terminal amino acid sequence of the Apple 3 domain (T^{249}−V^{271}) and is mediated in part by amino acid residues R^{250}, K^{255}, F^{260}, and Q^{263}.

Factor XI (FXI)[1] is a zymogen which contains two identical subunits, each of which can be cleaved at the internal R^{369}−I^{370} bond by factor XIIa (FXIIa) (*1*), thrombin, or factor XIa (FXIa) (*2*) to give rise to activated FXI. The in vitro activation of FXI by the contact activation pathway requires a negatively charged surface along with three additional contact activation proteins, factor XII (FXII), high-molecular weight kininogen (HK), and prekallikrein (PK) (*1*, *3*, *4*). However, persons congenitally deficient in FXII, HK, and PK do not exhibit abnormal bleeding (*2*), suggesting that these proteins are not required for coagulation in vivo. Alternatively, FXI can be activated independently of the contact activation proteins if thrombin or FXIa and a negatively charged surface, such as dextran sulfate, are present (*2*, *5*, *6*). However, the physiological correlation of this observation is unclear since dextran sulfate is not a component of surfaces exposed on cells or the subendothelial matrix in vivo.

Since the activation of FXI requires a surface (*2*, *5*−*7*), it is likely that in vivo, the most physiologically relevant sites involved in the initiation of the intrinsic or sustaining coagulation pathway are located on platelets. Thus, it has been shown that FXI binds specifically and reversibly to the surface of activated platelets ($K_d \sim 1.0 \times 10^{-8}$ M, $n \sim 1500$ sites/platelet) in the presence of HK, $ZnCl_2$, and $CaCl_2$ (*8*) or in the presence of prothrombin and $CaCl_2$ (*9*) and that the binding of FXI to activated platelets promotes the activation of FXI by FXIIa (*7*) or by thrombin (*9*), like the activation of FXI on dextran sulfate and other negatively charged surfaces (*2*, *5*, *6*). The interaction of FXI with platelets requires the binding of HK to the Apple 1 (A1) domain of FXI in the presence of Zn^{2+} ions (*10*) or the binding of prothrombin to the A1 domain of FXI in the presence of Ca^{2+} ions (*9*). The direct, high-affinity binding of FXI to platelets is mediated by a platelet binding site in FXI that has been localized to the sequence of amino acids N^{235}−R^{266} in the A3 domain of FXI (*9*, *11*). Given the physiological concentration of FXI in blood (∼30 nM), and the close correspondence of the level of FXI binding to platelets with rates of FXI activation by thrombin (*9*), the activated platelet surface is the likely site of initiation of surface-mediated intrinsic coagulation in vivo. This study was undertaken in an attempt to provide a detailed mapping of the platelet binding site in the A3 domain of FXI (FXI-A3).

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[1] Abbreviations: FXI, factor XI; FXIIa, factor XIIa; FXIa, factor XIa; FXII, factor XII; HK, high-molecular weight kininogen; PK, prekallikrein; A1−A4, Apple 1−4 domains, respectively; FXI-A3, A3 domain of FXI; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction; PK-A3, A3 domains of PK; FXa, factor Xa; FXI-A3U, upstream primer of FXI-A3; PK-A3U, upstream primer of PK-A3; FXI-A3D, downstream primer of FXI-A3; PK-A3D, downstream primer of PK-A3; FXI-A3D1, FXI-A3 downstream primer with a *Hin*dIII restriction site; rFXI, recombinant FXI; rPK, recombinant PK; rFXI-A3, recombinant Apple 3 domain of FXI; rPK-A3, recombinant Apple 3 domain of PK.

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EXPERIMENTAL PROCEDURES

Materials. Human FXI was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Restriction enzymes were from New England Biolabs (Beverly, MA). DNA markers, Pfu polymerase, ligase, deoxynucleotide triphosphate, and Sephadex G50 (fine) were from Pharmacia Biotech (Piscataway, NJ). The human FXI cDNA was a gift from D. Chung (University of Washington, Seattle, WA). Bacto tryptone and bacto yeast extract were from GIBCO/BRL (Grand Island, NY). Centricon 3, centricon 10, centricon 30, centriprep 3, centriprep 10, and centriprep 30 instruments were purchased form Amicon, Inc. (Beverly, MA). Trichloroacetic acid, β-mercaptoethanol, and disodium EDTA were purchased from Fisher Scientific (Springfield, NJ). Methyl silicon oil (1 DC-200) and Hi Phenyl silicon oil (125 DC-550) were purchased from William F. Nye, Inc. (Fairhaven, MA). Carrier-free $Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, IL). Goat anti-human FXI polyclonal IgG, with and without conjugated horseradish peroxidase, for the FXI enzyme-linked immunosorbent assay (ELISA) was from Enzyme Research Laboratories (South Bend, IN). Alkaline phosphatase-conjugated goat anti-mouse antibody was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Rabbit brain phospholipids (Cephalin) were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). The thrombin receptor agonist peptide SFLLRN-amide was synthesized on an Applied Biosystems (Foster City, CA) 430A synthesizer and via reverse-phase high-performance liquid chromatography (HPLC) purified to >99% homogeneity. Isopropyl β-D-thiogalactoside (IPTG) was purchased from LabScientific, Inc. (Princeton, NJ). 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 Laboratories, Inc. (Nelville, NY). Iodogen vials (20 μg coating) were obtained from Pierce (Rockford, IL). C18 Sep-Pak reversed-phase chromatographic cartridges were from Millipore (Milford, MA). Soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), aprotinin, benzamidine, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), sodium *m*-periodate, Sepharose 2B-CL, Sephadex G25 (fine), dithiothreitol, GuHCl, urea, dithionitrobenzoate (DTNB), sodium phosphate, Tris-HCl, Trizma base, cysteine, bovine serum albumin, iodoacetamide, and metabisulfite were purchased from Sigma Chemical Co. (St. Louis, MO).

Radiolabeling of FXI. Purified FXI was radiolabeled by a minor modification of the Iodogen method (*12*). Briefly, 2 μL of [^{125}I]NaOH (500−1000 μCi/μL) was added to 100 μL of FXI (0.1−1.0 mg/mL) and the mixture incubated in an Iodogen vial (20 μg coating) for 20 min. The reaction was stopped by adding metabisulfite (50 μg/mL). The [^{125}I]-FXI was purified by centrifugation at 200*g* for 20 min through a 1 mL Sephadex G50 column equilibrated with 0.01 M Tris (pH 8.0). The extent of incorporation of ^{125}I into FXI was ∼98%, and the specific radioactivity of the [^{125}I]FXI was 29.4 μCi/μg. This corresponds to 1.24 ± 0.40 atoms of ^{125}I per molecule of FXI monomer.

Peptide Synthesis. Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer by a modification of the procedure described by Kent and Clark-Lewis (*13*). All the peptides utilized in this work were rationally designed, conformationally constrained synthetic peptides based upon a previously published molecular model for the A3 domain of FXI (*11*). The designation of residues and sequences of the synthetic peptides were based on published residue numbers in FXI (*14*). The sequence designated N^{235}−R^{266} was ^{235}NLCLLKTSESGLPSTRIKKSKALSGF-SLQSCR^{266}. The sequences (with their designations in parentheses) of peptides, including cysteines, introduced for refolding were C^{229}PKESQ^{233}C (P^{229}−Q^{233}), C^{241}TSESGL^{246}C (T^{241}−L^{246}), C^{249}TSIKKSKALSGF^{260}C (T^{249}−F^{260},R^{250}S), C^{249}TRIPKSKALSGF^{260}C (T^{249}−F^{260},K^{252}P), C^{249}TRIK-QSKALSGF^{260}C (T^{249}−F^{260},K^{253}Q), C^{249}TRIKKSNALSGF^{260}C (T^{249}−F^{260},K^{255}N), C^{249}TRIKKSNAISGF^{260} (T^{249}−F^{260},L^{257}I), and C^{249}TRIKKSNTLSGF^{260} (T^{249}−F^{260},A^{256}T).

Folding of the Peptides. A previously published method (*15*) was used to refold peptides containing cysteine residues. Briefly, peptides were dissolved in deionized water to a final concentration of 0.1 mg/mL. The pH values of T^{249}−F^{260},R^{250}S; T^{249}−F^{260},K^{252}P; T^{249}−F^{260},K^{253}Q; T^{249}−F^{260},K^{255}N; T^{249}−F^{260},L^{257}I; and T^{249}−F^{260},A^{256}T were adjusted using 0.1 N NaOH to 9.30 and the mixtures stirred at 4 °C for 3 days.

Assay for Thiols. Synthetic or recombinant peptides at protein concentrations of 10 nM in 0.1 M phosphate buffer (pH 7.3) and 1 mM EDTA were added to 0.15 mM dithionitrobenzoate (DTNB) [in 0.1 M phosphate (pH 7.3)] and the mixtures incubated at room temperature for 10 min. A_{412} was monitored in a spectrophotometer for an additional 30 min or until absorbance stopped increasing. The molar concentrations of free thiols present were calculated from the molar absorbance of the nitrothiobenzoate anion (ϵ_{412} = 14 150 M^{-1} cm^{-1}) released from the reaction by the free thiols present in the peptides.

High-Performance Liquid Chromatography (HPLC). The HPLC system that was employed was from Waters (Waters 600 gradient module, model 740 data module, model 46K universal injector, and Lambda-Max model 481 detector, Milford, MA). Reverse-phase chromatography was performed using a Waters C8 μBondapak column, whereas gel filtration was carried out using a Waters Protein-Pak 60 column as previously described (*10*, *15*, *16*).

Characterization of Synthetic Peptides. All peptides utilized in this study were examined by HPLC (both reverse-phase and gel filtration), and all exhibited a single homogeneous peak (data not shown). When the refolded peptides were examined by HPLC (both reverse-phase and gel filtration), single peaks with retention times identical to those of the original mixtures were observed, demonstrating the presence of a single homogeneous mixture of refolded peptides.

Amplification and Transformation of Wild-Type and Mutant A3 Domains of Human FXI. Primers corresponding to the A3 domain were used in the polymerase chain reaction (PCR) with FXI or PK cDNA (a 2.1 kb *Eco*RI fragment containing the complete FXI or PK cDNA coding sequence, generous gifts from D. W. Chung, K. Fujikawa, and E. W. Davie of the Department of Biochemistry, University of Washington) to create a 303 bp insert that is identical to the A3 domain or the mutant A3 domains of FXI (FXI-A3) or PK (PK-A3). Primers were prepared by GIBCO BLR (Gaithersburg, MD). The upstream primer of FXI-A3 (FXI-

A3U) contains a *Bam*HI restriction site (underlined) and a cleavage site for factor Xa (FXa) (IEGR). Its sequence is 5′GCGGATCC(ATCGAGGGTAGA)GCTTGTATTAGGGACATTTTCCCT. The upstream primer of PK-A3 (PK-A3U) contains a *Bam*HI restriction site and a cleavage site for FXa (IEGR). Its sequence is 5′GCGGATCC(ATCGAGGGTAGA)GGTTGCCACATGAACATCTTCCAG. The downstream primer of FXI-A3 (FXI-A3D) contains a *Hin*dIII restriction site, and its sequence is 5′GCAGCTTTACACTGGGATGCTGTGCCTGCA. The downstream primer of PK-A3 (PK-A3D) contains a *Hin*dIII restriction site, and its sequence is 5′GCAGCTTTATTCAGGTAAAGTTCTTTTGCA.

The mutagenic primers are as follows: (1) rFXI-A3 $R^{250}A$, 5′TTGCCCAGTACAGCCATTAAAAAGAGC; (2) rFXI-A3 $K^{252}A$, 5′AGTACACGCATTGCAAAGAGCAAAGCT; (3) rFXI-A3 $K^{253}A$, 5′ACACGCATTAAAGCGAGCAAAGCTCTT; (4) rFXI-A3 $K^{255}A$, 5′ATTAAAAAGAGCGCAGCTCTTTCTGGT; (5) rFXI-A3 $F^{260}A$, 5′GCTCTTTCTGGTGCCAGTCTACAAAGC; and (6) rFXI-A3 $Q^{263}A$, 5′GGTTTCAGTCTAGCAAGCTGCAGGCAC. For PCR amplification of the wild type and mutant FXI-A3 and PK-A3, the procedures were based on a technique developed by Picard et al. (*17*).

For PCR amplification of the rPK−FXI-A3 chimera (in which the C-terminus of the FXI-A3, T^{249}−V^{271}, replaced the corresponding C-terminal end of the PK-A3, S^{249}−E^{271}), a FXI-A3 downstream primer (with a *Hin*dIII restriction site) was made, FXI-A3D1 (5′GCAAGCTTTACACTGGGATGCTGTGCCTGCAGCTTTGTAGACTGAAACCAGAAAGAGCTTTGCTCTTTTTAATGCGTGT). The procedure for PCR amplification of the chimera is as follows. One micromole of PK-A3U and 1 μmol of FXI-A3D1 primers are added to 10 ng of PK cDNA, 2.5 units of cloned Pfu DNA polymerase, 10 μL of 10× cloned Pfu buffer, and 0.2 mM nucleotide triphosphate to a final volume of 100 μL. The reaction mixture was overlaid with 100 μL of mineral oil. Samples were run in a thermocycler with 10 cycles of 1 min at 95 °C, 1 min at 48 °C, and 2 min at 72 °C, followed by 5 min at 72 °C, and finally held at 4 °C. After 10 cycles, an additional 20 μmol of PK-A3U and 20 μmol of FXI-A3D were added to the reaction mixture, which was reamplified according to the program as described above for another 30 cycles.

The PCR product was cut with *Bam*HI and *Hin*dIII restriction enzymes using the procedures supplied with the enzyme from New England Biolabs. The insert was ligated to the QIAexpress pQE-9 vector, and transformations were carried out using competent K-12-derived *Escherichia coli* M15 [pREP4] cells. Expression from the pQE-9 vector was induced by the addition of isopropyl β-D-thiogalactoside (IPTG).

Expression, Isolation, and Folding of the Recombinant A3 Domain. The procedures utilized to prepare wild-type rFXI-A3 and rPK-A3 and the rFXI-A3 mutants were described by Ho et al. (*18*).

Preparation of Washed Platelets. A 45 mL quantity of blood was collected in a 50 mL tube containing 5 mL of acid citrate dextrose (2.5% w/v trisodium citrate, 1.5% w/v citric acid, and 2.0% w/v dextrose). The blood was centrifuged at 800*g* for 20 min to obtain platelet-rich plasma. Platelet-rich plasma was removed to a new 50 mL tube and centrifuged again at 800*g* for 5 min to remove contaminating red blood cells. The nonpelleted material was centrifuged at 1800*g* for 15 min. The pellet was resuspended in 5 mL of HEPES Tyrode buffer (138 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 3.5 mM HEPES, 5.5 mM dextrose, and 0.38 mM $NaH_2PO_4 \cdot H_2O$) at pH 6.5 and gel filtered on a column of Sepharose 2B equilibrated in HEPES Tyrode buffer at pH 6.5. Platelets were counted using a Coulter Electronics particle counter (Hialeah, FL).

Activation of Washed Platelets. Platelets were activated with a peptide representing the amino terminus of the cleaved thrombin receptor (SFLLRN-amide). Platelets at a density of $\sim 10^8$ mL^{-1} were activated with 5 μM thrombin receptor peptide.

Coagulation Assays. The coagulation assays were performed using the kaolin-activated partial thromboplastin time. A master reaction mixture containing 50 μL of 25 mg of kaolin mixed with phospholipids (0.04% inosithin) in 5 mL of HEPES Tyrode buffer at pH 7.4 alone or with activated platelets (10^8 mL^{-1}) in HEPES-Tyrode buffer at pH 7.4, 50 μL of FXI deficient plasma, 1 nM FXI, and appropriate concentrations of rFXI-A3 or mutant rFXI-A3 proteins and HEPES Tyrode buffer at pH 7.4 were added to a final volume of 200 μL. The master mixture was incubated at 37 °C for 5 min; then 50 μL of 50 mM $CaCl_2$ was added, and the clotting time was determined using an Amelung KC4A Micro Coagulometer (Sigma Diagnostics, St. Louis, MO).

Platelet Binding Experiments. Binding and competition experiments were carried out as previously described (*8*). Briefly, platelets (10^8 mL^{-1}) were incubated with thrombin receptor peptide (5 μM), prepared as previously described (*8*), representing the amino terminus of the cleaved thrombin receptor (SFLLRN-amide) for 5 min at 37 °C. Then, the following reagents were added: 25 μM $ZnCl_2$, 2 mM $CaCl_2$, 50 nM HK, and various concentrations of [^{125}I]FXI in the presence or absence of various concentrations of either unlabeled FXI, rFXI-A3, rFXI, or conformationally constrained synthetic peptides. 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 pellets from the supernatant. The amount of [^{125}I]FXI bound to platelets was counted for ^{125}I using a Wallac 1470 Wizard gamma counter.

One hundred percent binding of [^{125}I]FXI represented an average of 250 000 cpm bound, whereas 0% binding represented background binding (250 cpm). In competition experiments, the concentration of competitor which displaced 50% of the bound [^{125}I]FXI (IC_{50}) was determined by plotting the amount of [^{125}I]FXI bound to platelets versus the amount of competitor ligand added. The K_i was calculated using the equation $IC_{50} = (1 + [S]/K_d)K_i$, where [S] is the concentration of [^{125}I]FXI used in these experiments (held constant at 22 nM) and K_d was the value ($\sim$10 nM) determined from direct binding experiments.

Conversion of the Inhibition Constant (K_i) to the Change in Binding Energy ($\Delta G°$). The $\Delta G°$ was calculated using the equation $\Delta G° = -RT \ln K_i$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}), T is the absolute temperature (310 K), and K_i is the concentration of inhibitor needed to displace 50% of the bound [^{125}I]FXI from 10^8 platelets/mL.

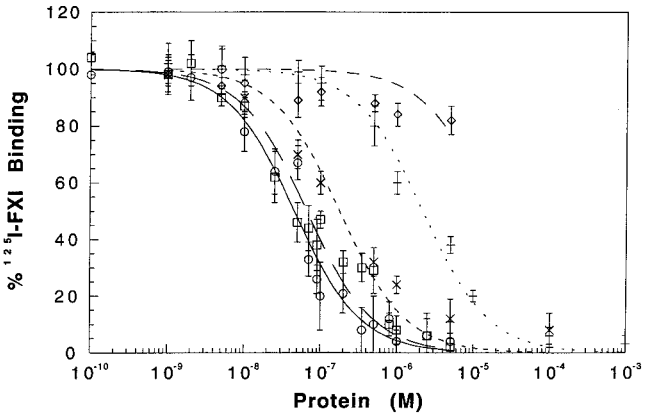

FIGURE 1: Effects of FXI, rFXI-A3 domains, and synthetic peptides on the binding of [^{125}I]FXI to platelets. The effects of competitive ligands such as unlabeled FXI (○), rFXI-A3 (□), rPK-A3 (◇), $N^{235}-R^{266}$ (×), and $T^{249}-F^{260}$ (+) on the binding of 22 nM [^{125}I]-FXI to activated platelets (1×10^8 platelets/mL), in the presence of HK (50 nM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM), and thrombin peptide (5 μM). The level of binding of [^{125}I]FXI to platelets was determined after incubation for 30 min at 37 °C with the indicated proteins at the indicated concentration. Binding of [^{125}I]FXI to platelets was assessed as described in Experimental Procedures. The level of binding of [^{125}I]FXI to platelets is the ratio of the level of binding of [^{125}I]FXI in the presence of the designated protein to the level of binding of [^{125}I]FXI in the absence of any competitive ligands.

Molecular Modeling. A molecular model of the A3 domain of FXI, constructed as previously described (*11*), was visualized utilizing a Silicon Graphics (Mountain View, CA) Octane computer and the Sybyl Software Package (Tripos Inc., St. Louis, MO).

RESULTS

Localization of the Platelet Binding Site in the FXI-A3 Domain. Competition experiments were conducted in which unlabeled FXI, the rFXI-A3, or a conformationally constrained synthetic peptide ($N^{235}-R^{266}$) was used to compete with [^{125}I]FXI for binding to activated platelets. Given the fact that 20 separate synthetic peptides and recombinant wild-type and mutant Apple domain molecules were examined in the work presented here, it would not have been feasible, nor would it have yielded reliable data, to radiolabel each ligand for direct binding measurements. Instead, competition binding studies were carried out based on the demonstration that the affinity and stoichiometry of FXI and rA3 domain binding are similar, that the binding was reversible, specific, and saturable, and that estimates of affinity were identical whether obtained from values of K_d in direct binding or from values of K_i in competition binding experiments (data not shown). These data are in agreement with the observation that a conformationally constrained A3 domain peptide $N^{235}-R^{266}$ (labeled with ^{125}I) binds to activated platelets with a stoichiometry (1035 sites/platelet) and an affinity ($K_d \sim 21$ nM) similar to those for FXI in a reversible and specific manner, either in the presence or in the absence of HK and $ZnCl_2$, and is displaced by unlabeled FXI in the presence of HK and $ZnCl_2$ (*11*). The rFXI-A3 had the same platelet binding affinity ($K_i \sim 6 \times 10^{-9}$ M; $\Delta G° \sim 11.6$ kcal/mol) as unlabeled FXI ($K_i \sim 5 \times 10^{-9}$ M; $\Delta G° \sim 11.8$ kcal/mol) which were similar to those of the $N^{235}-R^{266}$ peptide ($K_i \sim 4 \times 10^{-8}$ M; $\Delta G° \sim 10.5$ kcal/mol) (Figure 1 and Table 1).

Table 1: Effects of Various Proteins and Peptides on the Binding of [^{125}I]Factor XI to Platelets[a]

protein	K_i (M)	$\Delta G°$ (kcal/mol)
FXI (plasma-derived)	$(5 \pm 3) \times 10^{-9}$	11.8 ± 0.3
rFXI-A3	$(6 \pm 2) \times 10^{-9}$	11.6 ± 0.2
rPK−FXI-A3	$(8 \pm 4) \times 10^{-9}$	11.5 ± 0.4
rPK-A3	$(3 \pm 2) \times 10^{-6}$	7.8 ± 0.2
rFXI-A3R^{250}A	$(5 \pm 1) \times 10^{-8}$	10.4 ± 0.7
rFXI-A3K^{252}A	$(7 \pm 2) \times 10^{-9}$	11.6 ± 0.2
rFXI-A3K^{253}A	$(7 \pm 4) \times 10^{-9}$	11.6 ± 0.4
rFXI-A3K^{255}A	$(3 \pm 2) \times 10^{-8}$	10.7 ± 0.2
rFXI-A3F^{260}A	$(9 \pm 3) \times 10^{-8}$	9.9 ± 0.3
rFXI-A3Q^{263}A	$(2 \pm 1) \times 10^{-8}$	10.9 ± 0.5
$N^{235}-R^{266}$	$(4 \pm 1) \times 10^{-8}$	10.5 ± 0.5
$P^{229}-Q^{233}$	$(1 \pm 1) \times 10^{-4}$	5.7 ± 0.5
$T^{241}-L^{246}$	$(4 \pm 2) \times 10^{-4}$	4.8 ± 0.2
$T^{249}-F^{260}$	$(7 \pm 3) \times 10^{-7}$	8.7 ± 0.3
$T^{249}-F^{260}$,R^{250}S	$(1 \pm 1) \times 10^{-4}$	5.7 ± 0.5
$T^{249}-F^{260}$,K^{252}P	$(3 \pm 2) \times 10^{-6}$	7.8 ± 0.2
$T^{249}-F^{260}$,K^{253}Q	$(2 \pm 2) \times 10^{-7}$	9.5 ± 0.2
$T^{249}-F^{260}$,K^{255}N	$(4 \pm 1) \times 10^{-7}$	9.1 ± 0.6
$T^{249}-F^{260}$,A^{256}T	$(3 \pm 2) \times 10^{-7}$	9.3 ± 0.2
$T^{249}-F^{260}$,L^{257}I	$(3 \pm 3) \times 10^{-7}$	9.3 ± 0.3

[a] For the rFXI mutants (residues 250−255 → A), only the basic amino acids at positions 250, 252, 253, and 255 are changed to alanine. The amino acids at positions 251 and 254 are the same as wild-type FXI.

FXI ^{235}N L C L L K T S E S G L P S T R I K K S K A L S G F S L Q S C R

PK ^{235}N V C L L K T S E S G T P S S S T P Q E N T I S G Y S L L T C K

FIGURE 2: Comparison of amino acid sequences of the A3 domains of FXI and PK. Sequences of the underlined amino acids indicate that the cluster of these basic amino acids, in the FXI-A3 domain, may play an important role in platelet binding.

Furthermore, neither the rFXI-A3 domain nor any peptide derived from the A3 domain was able to bind to HK or to FXI itself (data not shown) in solid-phase assays described previously (*10*, *19*, *20*). Therefore, the A3 domain of FXI and peptides derived from it interact directly with the platelet surface (and not with either HK or FXI) to block binding of FXI to activated platelets. We conclude that all the energy required for FXI binding to activated platelets resides within the A3 domain, mainly within residues $N^{235}-R^{266}$.

To refine the FXI platelet binding site, the amino acid sequence of the A3 domain of FXI was compared with that of PK (Figure 2), since the sequences of these two proteins are 58% identical and each has four Apple domains per subunit (*14*, *21*). In contrast to the rFXI-A3 domain and its peptide ($N^{235}-R^{266}$), neither the rPK-A3 nor its A3 domain peptide ($N^{235}-K^{266}$), which is 52% identical to the FXI A3 domain peptide ($N^{235}-R^{266}$), is capable of competing with [^{125}I]FXI for binding to platelets (*11*) (Figure 1). Comparison of FXI and PK shows that a sequence between residues 249 and 260 (Figure 2, underlined) is entirely different in these two proteins. This region of 12 amino acids might therefore comprise the major platelet binding site in FXI. To test this hypothesis, a conformationally constrained synthetic peptide ($T^{249}-F^{260}$) was constructed that encompassed these amino acids and was used in the competition binding experiment. The result from this study suggested that the amino acid sequence $T^{249}-F^{260}$ contained a portion of the platelet binding site ($K_i \sim 7 \times 10^{-7}$ M; $\Delta G° \sim 8.7$ kcal/mol) (Figure 1 and Table 1). In contrast, two other conformationally constrained peptides, $P^{229}-Q^{233}$ and $T^{241}-L^{246}$, comprising sequences within the A3 domain of FXI competed with [^{125}I]FXI for

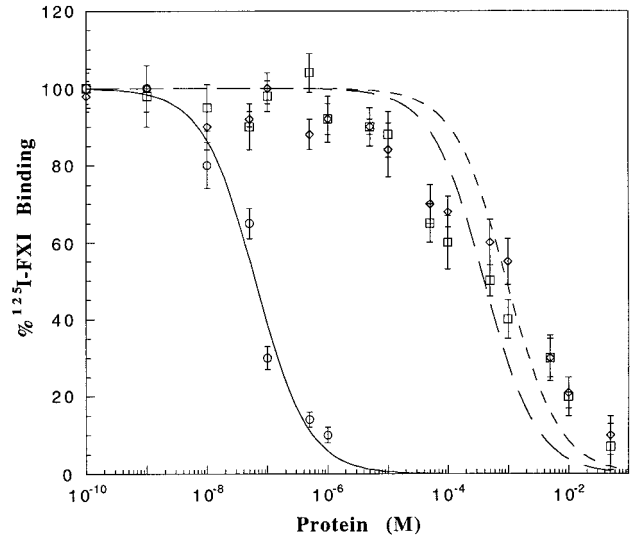

FIGURE 3: Effects of the rPK−FXI-A3 chimera and P^{229}−Q^{233} and T^{241}−L^{246} synthetic peptides on the binding of [^{125}I]FXI to platelets. The effects of competitive ligands such as the the rPK−FXI-A3 chimera (○), P^{229}−Q^{233} (□), and T^{241}−L^{246} (◇) on the binding of 22 nM [^{125}I]FXI to activated platelets (10^8 platelets/mL), in the presence of HK (50 nM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM), and thrombin peptide (5 μM). The level of binding of [^{125}I]FXI to platelets was determined after incubation for 30 min at 37 °C with the indicated proteins at the indicated concentration. The levels of binding of [^{125}I]FXI to platelets were determined as described in Experimental Procedures. The level of binding of [^{125}I]FXI to platelets is the ratio of the level of binding of [^{125}I]FXI in the presence of the designated protein to the level of binding of [^{125}I]-FXI in the absence of any competitive ligands.

binding to activated platelets very poorly (P^{229}−Q^{233}, $K_i \sim 1 \times 10^{-4}$ M; T^{241}−L^{246}, $K_i \sim 4 \times 10^{-4}$ M) (Figure 3 and Table 1).

To test the hypothesis that the platelet binding site may extend beyond the C-terminus of the peptide T^{249}−F^{260}, we constructed a potential "gain-of-function" chimera (rPK−FXI-A3) in which the C-terminus of the FXI-A3 (T^{249}−V^{271}) replaced the corresponding C-terminal end of the PK-A3 (S^{249}−E^{271}). A competition experiment was carried out in which we examined the capacity of the rPK−FXI-A3 to displace [^{125}I]FXI for binding to activated platelets. The rPK−FXI-A3 chimera was able to compete with [^{125}I]FXI for binding to the platelet surface ($K_i \sim 8 \times 10^{-9}$ M) (Figure 3 and Table 1) with an affinity similar to that of the rFXI-A3 ($K_i \sim 6 \times 10^{-9}$ M) (Figure 1). This experiment suggests that FXI binding to activated platelets is mediated exclusively by the amino acids at the C-terminus (T^{249}−V^{271}) of the A3 domain of FXI.

Fine Mapping of the Platelet Binding Site on the rFXI-A3. Competition binding studies were also carried out with synthetic peptides in which specific amino acid substitutions from FXI to PK residues were introduced to provide clues concerning the residue(s) that may be important for binding to platelets in the A3 domain of FXI (Figure 4A and Table 1). A $R^{250} \rightarrow$ S substitution disrupted the ability of the peptide T^{249}−F^{260} to compete with [^{125}I]FXI for binding to activated platelets ($K_i \sim 1 \times 10^{-4}$ M), whereas the $K^{252} \rightarrow$ P ($K_i \sim 3 \times 10^{-6}$ M) and the $K^{255} \rightarrow$ N ($K_i \sim 4 \times 10^{-7}$ M) substitutions exhibited quantitatively smaller defects. The other "homologue scanning" peptides exhibited no deficiency in their ability to compete with [^{125}I]FXI for binding to platelets: T^{249}−F^{260},K^{253}Q, $K_i \sim 2 \times 10^{-7}$ M; T^{249}−F^{260},A^{256}T, $K_i \sim 3 \times 10^{-7}$ M; and T^{249}−F^{260},L^{257}I, $K_i \sim 3 \times 10^{-7}$ M (Table 1).

To further refine the characteristics of the platelet binding site within the A3 domain of FXI, we utilized the information obtained from the synthetic peptide studies which identified R^{250} and K^{252} as possibly being important in platelet binding. Examination of a molecular model of the FXI-A3 domain (*11*) revealed that the side chains of amino acids R^{250}, K^{255}, F^{260}, and Q^{263} were projected on one surface of the A3 domain whereas the side chains of K^{252} and K^{253} were

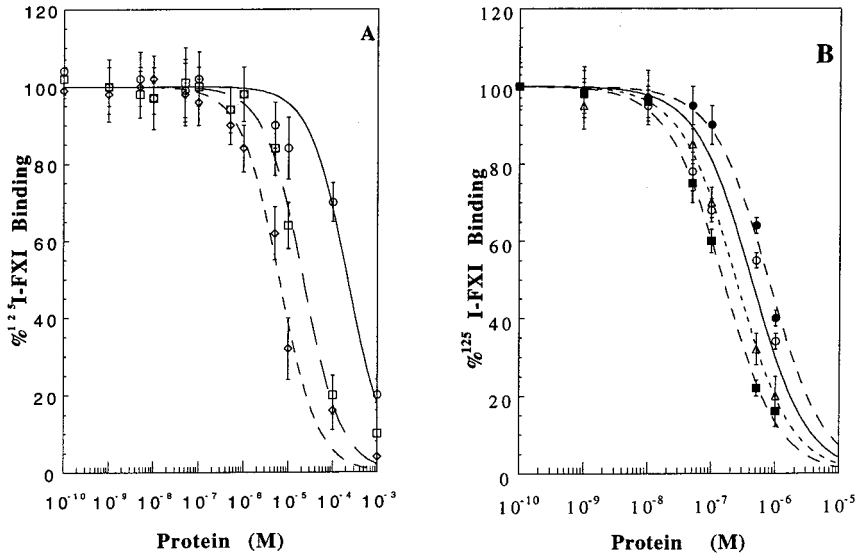

FIGURE 4: Fine mapping of the platelet binding site using the synthetic peptides and rFXI-A3. The effects of competitive ligands such as (A) T^{249}−F^{260},R^{250}S (○), T^{249}−F^{260},K^{252}P (□), and T^{249}−F^{260},K^{255}N (◇) and (B) rFXI-A3R^{250}A (○), rFXI-A3K^{255}A (△), rFXI-A3F^{260}A (●), and rFXI-A3Q^{263}A (■) on the binding of 22 nM [^{125}I]FXI to activated platelets (1×10^8 platelets/mL), in the presence of HK (50 nM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM), and thrombin peptide (5 μM). The level of binding of [^{125}I]FXI to platelets was determined after incubation for 30 min at 37 °C with the indicated proteins at the indicated concentration. The levels of binding of [^{125}I]FXI to platelets were determined as described in Experimental Procedures. The level of binding of [^{125}I]FXI to platelets is the ratio of the level of binding of [^{125}I]FXI in the presence of the designated protein to the level of binding of [^{125}I]FXI in the absence of any competitive ligands.

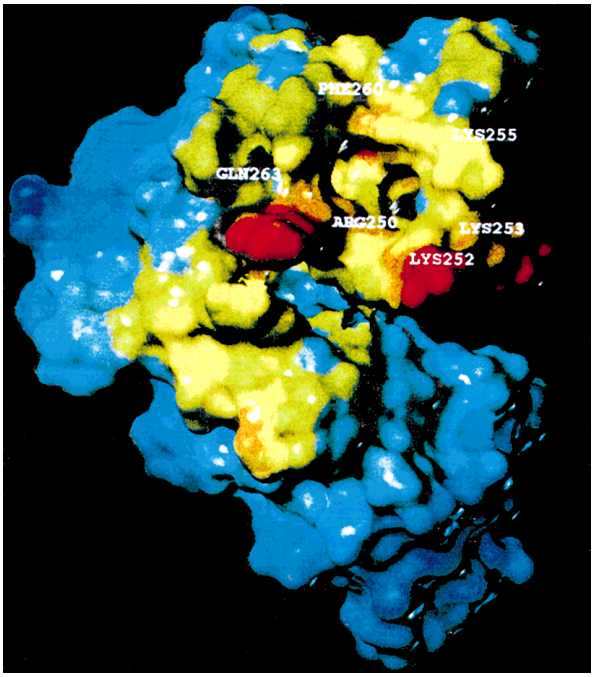

FIGURE 5: Molecular model of the surface of the rFXI-A3. The solvent-exposed surface of the molecular model (*11*) of the rFXI-A3 shows residues K^{252} and K^{253}, which are important for heparin binding, displayed on the right side of the model. The residues identified in this paper as being important for binding of FXI to platelets (R^{250}, K^{255}, F^{260}, and Q^{263}) are shown in a cluster hypothesized to comprise the platelet binding site.

projected toward another surface (Figure 5). Using this information, a series of mutated rFXI-A3 proteins were made by alanine substitution of amino acid residues R^{250}, K^{252}, K^{253}, K^{255}, F^{260}, and Q^{263}. To refine the mapping of the platelet binding site in the A3 domain of FXI, competition binding experiments were conducted in which these rFXI-A3 mutants were allowed to compete against [^{125}I]FXI for binding to activated platelets. The results (Figure 4B and Table 1) indicate that R^{250}, K^{255}, F^{260}, and Q^{263} are important residues for platelet binding (rFXI-A3F^{260}A, $K_i \sim 9 \times 10^{-8}$ M, $\Delta G° \sim 9.9$ kcal/mol; rFXI-A3R^{250}A, $K_i \sim 5 \times 10^{-8}$ M, $\Delta G° \sim 10.4$ kcal/mol; rFXI-A3K^{255}A, $K_i \sim 3 \times 10^{-8}$ M, $\Delta G° \sim 10.7$ kcal/mol; rFXI-A3Q^{263}A, $K_i \sim 2 \times 10^{-8}$ M, $\Delta G° \sim 10.9$ kcal/mol). Amino acids K^{252} and K^{253}, which are implicated as being important for heparin binding (*18*), did not appear to be important for the binding of FXI-A3 to platelets (rFXI-A3K^{252}A, $K_i \sim 7 \times 10^{-9}$ M, $\Delta G° \sim 11.6$ kcal/mol; rFXI-A3K^{253}A, $K_i \sim 7 \times 10^{-9}$ M, $\Delta G° \sim 11.6$ kcal/mol) (Table 1).

Effects of Mutant rFXI-A3 Proteins in Coagulation Assays Containing Platelets or Phospholipids. Previous studies from our laboratory showed that the synthetic peptide N^{235}–R^{266} inhibited FXI coagulant activity when platelets were used as a surface but failed to inhibit FXI activity when phospholipids were used as a surface (*11*). Since the A3 domain of FXI encompasses the sequence N^{235}–R^{266} and contains the platelet binding site, we carried out partial thromboplastin time coagulation assays to determine the effects of the rFXI-A3 and its mutants on their ability to inhibit platelet surface-mediated coagulation by FXI. The results presented in Table 2 show the concentrations of each peptide required to inhibit FXI coagulation activity by 50% (IC_{50}). When phospholipids (0.04% inositin) were used as a surface, neither the native

Table 2: Effects of the Wild-Type and Mutant Recombinant Apple 3 Domains of Factor XI on Clotting Activity

protein	IC_{50} (M) 10^8 platelets/mL	IC_{50} (M) phospholipids
rFXI-A3	$(5 \pm 2) \times 10^{-8}$	$(6 \pm 4) \times 10^{-6}$
rPK−FXI-A3	$(5 \pm 4) \times 10^{-8}$	$(5 \pm 3) \times 10^{-6}$
rPK-A3	$(4 \pm 3) \times 10^{-6}$	$(1 \pm 2) \times 10^{-5}$
rFXI-A3R^{250}A	$(9 \pm 1) \times 10^{-7}$	$(2 \pm 2) \times 10^{-5}$
rFXI-A3K^{252}A	$(6 \pm 2) \times 10^{-8}$	$(8 \pm 2) \times 10^{-6}$
rFXI-A3K^{253}A	$(8 \pm 4) \times 10^{-8}$	$(1 \pm 1) \times 10^{-5}$
rFXI-A3K^{255}A	$(7 \pm 2) \times 10^{-7}$	$(1 \pm 1) \times 10^{-5}$
rFXI-A3F^{260}A	$(2 \pm 1) \times 10^{-6}$	$(1 \pm 1) \times 10^{-5}$
rFXI-A3Q^{263}A	$(4 \pm 3) \times 10^{-7}$	$(1 \pm 1) \times 10^{-5}$

rFXI-A3 domain nor its mutants were able to inhibit FXI activity except partially at concentrations of $>10^{-6}$ M. In contrast, when platelets were used as a surface, the wild-type rFXI-A3 domain was a potent inhibitor of FXI activity ($IC_{50} \sim 5 \times 10^{-8}$ M), whereas the rPK-A3 ($IC_{50} \sim 1 \times 10^{-6}$ M) was 2 orders of magnitude less effective. Additionally, mutants rFXI-A3F^{260}A ($IC_{50} \sim 2 \times 10^{-6}$ M), rFXI-A3R^{250}A ($IC_{50} \sim 9 \times 10^{-7}$ M), rFXIA3K^{255}A ($IC_{50} \sim 7 \times 10^{-7}$ M), and rFXI-A3Q^{263}A ($IC_{50} \sim 4 \times 10^{-7}$ M) were defective in their capacity to inhibit FXI coagulant activity, whereas the two mutants shown to be normal in binding to platelets (rFXI-A3K^{252}A and rFXI-A3K^{253}A) were entirely normal in their capacity to inhibit platelet-mediated FXI activity. Collectively, these experiments indicate that FXI interacts specifically with the platelet surface through the A3 domain and that the binding of FXI to platelets is required for platelet-mediated F-XI activation of blood coagulation. The results of these experiments are summarized in Table 2.

DISCUSSION

The purpose of the experiments reported here was to characterize the platelet binding site within the A3 domain of blood coagulation FXI (FXI) that mediates the interaction of FXI with activated platelets in the presence of HK, $ZnCl_2$, and $CaCl_2$ (*8*, *9*, *11*) and promotes the activation of platelet-bound FXI by thrombin (*5*, *6*, *9*), by FXIa (*5*, *6*, *9*), or by FXIIa (*3*, *7*). The activated platelet surface is the most physiologically relevant site of F-XI activation by thrombin, generated in trace amounts by proteolytic activation of prothrombin by FXa (*22*, *23*). The initial burst of FXa arises from the activation of FX by the FVIIa−tissue factor complex before it is quickly shut down by the tissue factor pathway inhibitor (*24*, *25*). Thereby, the initial event in the activation of the intrinsic pathway of blood coagulation would appear to occur via a positive feedback reaction of thrombin generated by the tissue factor pathway rather than via the activation of the contact pathway involving proteins (i.e., HK, FXII, and PK), the deficiencies of which are not associated with defective hemostasis (*2*).

The methodological approach utilized in the study presented here to characterize the platelet binding site combined a number of techniques, including molecular modeling of protein structure (*11*), the use of rationally designed, conformationally constrained synthetic peptides (*11*), and both alanine and homologue scanning mutagenesis of rFXI-A3.

The secondary and tertiary structures of the FXI-A3 domain were predicted using molecular modeling (*11*). This

model (Figure 5) was used to predict the solvent accessible surfaces possibly comprising the platelet binding site on the FXI A3 domain. Conformationally constrained synthetic peptides with solvent-exposed surfaces suggested by the molecular model were prepared for these studies. In competition binding experiments, the peptide $N^{235}-R^{266}$ competed with [^{125}I]FXI for binding to activated platelets ($K_i \sim 40$ nM) with an affinity higher than that of the peptide $T^{249}-F^{260}$ ($K_i \sim 700$ nM) but very similar to that of unlabeled FXI ($K_i \sim 5$ nM) (Figure 1 and Table 1). Since the K_i for the peptide $T^{249}-F^{260}$ is approximately 8- and 17-fold higher than the respective K_i values for unlabeled FXI and for the peptide $N^{235}-R^{266}$, it is likely that the peptide $T^{249}-F^{260}$ contained part of the platelet binding energy and that additional binding energy might reside elsewhere within the A3 domain.

Previously, Baglia et al. (*11*) reported that peptides $P^{229}-Q^{233}$ and $T^{241}-L^{246}$ synergistically contributed to the overall platelet binding. Nevertheless, in binding competition studies, the effects of these peptides on the [^{125}I]FXI binding to activated platelets were relatively poor and did not follow the model of strict competitive inhibition (Figure 3 and Table 1). Thus, it is highly probable that whereas the peptide $T^{249}-F^{260}$ contains the major platelet binding energy, the peptides $P^{229}-Q^{233}$ and $T^{241}-L^{246}$ may play a role in stabilizing the overall conformation of the $T^{249}-F^{260}$ to optimize platelet binding (*11*). Thus, the peptide $T^{249}-F^{260}$ is not a completely effective competitive inhibitor of FXI binding to activated platelets since it does not contain all of the platelet binding energy. Nevertheless, the utilization of the peptide $T^{249}-F^{260}$ as a probe to fine map the platelet binding site on the FXI-A3 domain was useful for our subsequent experiments.

Since PK has no capacity to bind to activated platelets, to displace peptide [^{125}I]$N^{235}-R^{266}$ from platelet binding sites (*11*), or to compete with FXI for platelet binding (*8*) and since the amino acid sequence of the A3 domain of PK is 53% identical to the FXI-A3 domain but entirely nonidentical within the sequence of amino acids of residues 249−257 (Figure 2), we employed homologue scanning mutagenesis to replace individual amino acids in the $T^{249}-F^{260}$ region of FXI with the corresponding amino acids from PK to map the specific amino acid residues that are required for platelet binding within the $T^{249}-F^{260}$ region. Homologue scanning mutagenesis has been successfully used to identify the region of a G-protein mediating binding to adenylyl cyclase (*26*). Since the amino acid substitutions are from a highly homologous protein, the mutations are less likely than random mutations to disrupt the global conformation of the protein or peptide. A caveat to this argument is that certain amino acid substitutions can affect the backbone structure of a protein, such as the replacement of K^{252} with proline. Since proline characteristically results in turn formation in the peptide backbone, this substitution may produce a "kink" in the mutant peptide $T^{249}-F^{260}$,$K^{252}P$. The results from the homology scanning studies suggested that residues R^{250}, K^{252}, and K^{255} may be important for platelet binding (Figure 4A). The diminished platelet binding capacity of mutant peptides $T^{249}-F^{260}$,$R^{250}S$ and $T^{249}-F^{260}$,$K^{255}N$ may be due to the removal of amino acids that interact with a platelet receptor (R^{250} and K^{255}), whereas disruption of peptide conformation may result from the $K^{252}P$ substitution.

Since neither the synthetic peptide $T^{249}-F^{260}$ nor $N^{236}-R^{266}$ contains all of the platelet binding activity (Figure 1 and Table 1), it is possible that additional platelet binding energy resides elsewhere within the FXI-A3 domain or, alternatively, that the conformation of the synthetic peptides may prevent them from binding effectively to platelets. For these reasons, the rFXI-A3 and rPK-A3 domains were prepared and examined for platelet binding activity. The results of the competition studies indicate that the platelet binding site resides exclusively within the FXI-A3 domain and further verifies that the PK-A3 domain is unable to interact with the platelet FXI binding site (Figure 1 and Table 1). We then constructed a chimera (rPK−FXI-A3) in which the C-terminus of the PK-A3 domain was removed and replaced with the amino acids at the C-terminus of FXI-A3 domain ($T^{249}-V^{271}$). The purpose of this experiment was to determine whether the platelet binding site resides within the C-terminus of the FXI-A3 (as suggested by experiments with synthetic peptides whose results are presented in Figure 1 and Table 1) and to examine the possible contributions of subdomains $P^{229}-Q^{233}$ and $T^{241}-L^{246}$ to platelet binding. The underlying assumption for the construction of the rPK−FXI-A3 chimera is that the overall structure of the chimeric molecule should be similar to those of rPK and rFXI. Although the three-dimensional structures of PK-A3 and FXI-A3 are unknown, the primary amino acid sequences of the two proteins are highly conserved [58% identical (*14*)], suggesting that they are evolutionarily related proteins. Studies conducted by Craik et al. (*27*) on protein structures have shown that the crystal structures for evolutionarily related proteins are very similar. Moreover, the backbone structures of many serine proteases, which are evolutionary related, are alike with the major differences found in the surface-exposed residues that mediate ligand interactions (*28*). Therefore, if the residues mediating platelet binding reside exclusively at the C-terminus of the FXI-A3 domain, the gain-of-function rPK−FXI-A3 chimera should possess normal binding activity. The capacity of rPK−FXI-A3, when used in a competition experiment, to compete with [^{125}I]FXI for binding to activated platelets (Figure 3 and Table 1) was the same as that of the native rFXI-A3 (Figure 1 and Table 1), strongly supporting the conclusion that all the platelet binding energy of FXI resides at the C-terminus of the A3 domain within residues $T^{249}-V^{271}$ and that the peptide sequences $P^{229}-Q^{233}$ and $T^{241}-L^{246}$ have no direct role in platelet binding.

Since the platelet binding subdomain is localized within the C-terminal region of the FXI-A3 domain ($T^{249}-V^{271}$) and the homologue scanning mutagenesis studies of conformationally constrained synthetic peptides identified amino acid residues R^{250} and K^{255} as being important for platelet binding, we examined our molecular model (*11*) of the FXI-A3 domain (Figure 5). This model suggests that the side chains of residues R^{250}, K^{255}, F^{260}, and Q^{263} are projected in a cluster on one face of the surface of the A3 domain, whereas the side chains of residues K^{252} and K^{253} are projected on a different face (Figure 5). A caveat of the interpretation of this molecular model is that it is not based on any known structural information, and therefore, the three-dimensional spacial relationships predicted by the model are unlikely to be correct in detail. However, this and similar models have been powerful predictors of the location and conformation

of subdomains of the Apple domains of FXI involved in binding HK (*10*), thrombin (*29*), FIX (*30*), platelets (*11*), heparin (*18*), and FXIIa (*16*). We believe this record of success justifies a consideration of the model in the design of mutagenesis experiments. This suggests the possibility that the heparin binding surface, consisting at least in part of K^{252} and K^{253} (*18*), is projected on one face, whereas the platelet-binding surface is projected on an opposing albeit contiguous face. To test this hypothesis and to fine map the platelet binding site on the FXI-A3 domain, we chose to substitute amino acid residues R^{250}, K^{252}, K^{253}, K^{255}, F^{260}, and Q^{263} with alanine in isolated rFXI-A3 domain constructs. These conservative alanine substitutions should permit the effective removal of the side chains to be evaluated while minimizing the disruption in the secondary and tertiary conformation of the protein (*31*, *32*). The results from the rFXI-A3 mutagenesis studies reveal that while residues K^{252} and K^{253} are important for heparin binding (*18*), these residues play no role in platelet binding (Table 1). However, residues R^{250}, K^{255}, F^{260}, and Q^{263} are essential for normal platelet binding (Figure 4B and Table 1). The free energies of binding of FXI, the rFXI-A3 domain, and the rPK−FXI-A3 chimera were shown to be virtually identical ($\Delta G° = 11.5-11.8$ kcal/mol), whereas that calculated for the rPK-A3 domain was only 7.8 kcal/mol, a calculated deficit of ~4.0 kcal/mol (Table 1). The sum of the calculated deficits resulting from alanine substitutions at the four residues identified by our analysis is 4.5 kcal/mol, i.e., $\Delta\Delta G°$ values of 1.2 kcal/mol for rFXI-A3R^{250}A, 0.9 kcal/mol for rFXI-A3K^{255}A, 1.7 kcal/mol for rFXI-A3F^{260}A, and 0.7 kcal/mol for rFXI-A3Q^{263}A. Therefore, we conclude that virtually all the difference between FXI and PK in platelet binding energy can be ascribed to residues R^{250}, K^{255}, F^{260}, and Q^{263}.

An important conclusion that can be extracted from this study is the fact that the binding of FXI to activated platelets, mediated by amino acids at the C-terminus of the FXI A3 domain, is essential for in vitro clotting activity in the presence of activated platelets but not phospholipids. In the activated partial thromboplastin time test, using activated platelets as a binding surface, the rFXI-A3R^{250}A, rFXI-A3K^{255}A, rFXI-A3F^{260}A, and the rFXI-A3Q^{263}A mutants failed to inhibit FXI activity normally when compared to the wild-type rFXI-A3 and the rPK−FXI-A3 chimera, whereas the two mutants (rFXI-A3K^{252}A and rFXI-A3K^{253}A), which were shown to be normal in platelet binding, provided an inhibitory activity that was similar to that of the native rA3 (Table 2).

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