Prothrombin Is a Cofactor for the Binding of Factor XI to the Platelet Surface and for Platelet-Mediated Factor XI Activation by Thrombin†

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ABSTRACT: To study the physiological significance of thrombin as an initiator of intrinsic blood coagulation, activated human platelets were compared with dextran sulfate as a surface for thrombin-catalyzed factor XI activation. Activated gel-filtered platelets promoted factor XI activation by thrombin at initial rates 2−5-fold greater than dextran sulfate in the presence of high molecular weight kininogen (HK, 45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM), conditions optimal for factor XI binding to platelets. Physiological concentrations of HK (636 nM) inhibited factor XI activation by thrombin in a concentration-dependent manner, and this inhibition was reversed by prothrombin (1−3 μM) and by prothrombin fragment 1.2 (PF1.2), but not by prothrombin fragment 1 (PF1). Since prothrombin and PF1.2 (but not PF1) also displaced HK from its binding site on the Apple 1 domain of factor XI, we conclude that the Kringle II domain of prothrombin competes with HK for binding to the Apple 1 domain of factor XI. Prothrombin (1−3 μM) and PF1.2 (but not PF1) in the presence of $CaCl_2$ (2 mM) were able to replace HK (45 nM) in the presence of $ZnCl_2$ (25 μM) as a cofactor for the specific, reversible, high-affinity (K_d ∼25 nM) binding of factor XI to 947 ± 150 sites per platelet. This binding is mediated by residues Asn 235−Arg 266 in the Apple 3 domain since a conformationally constrained, synthetic peptide analogue of this sequence inhibits both factor XI binding to activated platelets and platelet-mediated, thrombin-catalyzed factor XI activation in the presence of prothrombin and $CaCl_2$. Finally, prothrombin (1.2 μM) and $CaCl_2$ (2 mM) could substitute for HK (45 nM) and $ZnCl_2$ (25 μM) in promoting optimal rates of thrombin-catalyzed factor XI activation on the platelet surface, thereby initiating the intrinsic coagulation pathway by mechanisms completely independent of the contact phase proteins, factor XII, HK, and prekallikrein.

Plasma coagulation factor XI is a unique disulfide-linked homodimer consisting of two identical polypeptide chains each of which can be proteolytically activated by factor XIIa, by factor XIa, or by thrombin by cleavage at an internal Arg 369−Ile 370 bond to give rise to factor XIa *(1−9).* This glycoprotein circulates in plasma as a complex with its cofactor high molecular weight kininogen (HK)[1] *(10, 11).* Activated platelets in the presence of HK bind factor XI reversibly and specifically to high-affinity sites on the surface of stimulated human platelets in the presence of Zn^{2+} and Ca^{2+} ions *(12).* The functional consequence of factor XI binding to activated platelets is an acceleration of the rate of factor XI activation by factor XIIa *(13)* similar to that which occurs in the presence of negatively charged surfaces such as kaolin, Celite, or dextran sulfate *(1−3).* The binding of factor XI to platelets is mediated by a sequence of amino acids (Asn 235−Arg 266) in the Apple 3 (A3) domain of factor XI that binds to activated platelets in a specific, reversible, and saturable manner *(14).*

It has been suggested that small quantities of thrombin, generated via the tissue factor pathway, may activate factor XI in vivo, thereby activating the intrinsic pathway of coagulation by a mechanism independent of the "contact phase" proteins, factor XII, prekallikrein, and HK *(8, 9).* Two potential objections to this suggestion are as follows: (1) the activation of factor XI by potentially physiological concentrations of thrombin requires the presence of dextran sulfate or similar negatively charged substances not present in vivo *(8, 9);* and (2) physiological concentrations of HK inhibit thrombin-mediated factor XI activation and effectively shut down this pathway *(8, 9, 15, 16).* On the other hand, recent experiments by von dem Borne and co-workers suggest that factor XI is activated by thrombin to generate antifibrinolytic activity at the site of clot formation even in the absence of a surface *(17, 18).* We have recently reported that the A1 domain of factor XI contains contiguous binding sites for HK and thrombin which may explain why HK at physiological concentrations inhibits thrombin-catalyzed factor XI activation *(19).* Therefore, one objective of the present study was to determine the mechanism by which HK inhibits thrombin-catalyzed factor XI activation and to ascertain

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[1] Abbreviations: HK, high molecular weight kininogen; A1, Apple 1; PF1, prothrombin fragment 1; PF1.2, prothrombin fragment 1.2; PPACK, prolylphenylalanylarginyl chloromethyl ketone; HPLC, high-performance liquid chromatography.

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whether physiologically relevant mechanisms exist by which this inhibitory effect of HK might be obviated. The marked enhancement of factor XI activation by thrombin in the presence of dextran sulfate *(8, 9)* suggests that proteoglycan or glycosaminoglycan or biochemically similar binding sites present on a cell or platelet surface may be required for optimal activation in vivo. Thus, another goal of this study was to determine whether activated platelets can also promote the activation of factor XI by thrombin.

Four repeat sequences (designated A1, A2, A3, and A4 or Apple domains) are present in the heavy-chain region of factor XI *(4, 5)*. Their primary structure has been elucidated from the sequence of a cDNA insert coding for factor XI *(4)*. We have previously reported evidence of the presence of HK and thrombin binding sites in the A1 domain, a substrate binding site for factor IX in the A2 domain, a binding site for factor XIIa in the A4 domain, and a platelet binding site in the A3 domain *(14, 19−23)*. In this study, we provide experimental evidence that supports the conclusion that factor XI and prothrombin form a complex that promotes factor XI binding to the platelet surface and its activation by thrombin.

EXPERIMENTAL PROCEDURES

Purification of Proteins. Factor XI (250 units/mg of protein) was purified from human plasma by immunoaffinity chromatography *(24)*. Factor XI was assayed by minor modifications *(25)* of the kaolin-activated partial thromboplastin time *(26)*. Human prothrombin, prothrombin fragment 1 (PF1), and prothrombin fragment 1.2 (PF1.2) were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). HK (specific activity 15 units/mg) was purified by the method of Kerbiriou and Griffin *(27)*. Human α-thrombin (2 800 NIH units/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). The potent thrombin inhibitor, prolylphenylalanylarginyl chloromethyl ketone (PPACK), was purchased from Calbiochem (Indianapolis, IN). Active-site-inhibited thrombin was prepared by incubation of a 10-fold excess of PPACK with α-thrombin for 1 h at 37 °C, and this mixture was dialyzed in Spectrophor tubing (3 500 M_r cutoff, Spectrum Medical Industries, Los Angeles, CA) overnight in PBS at 5 °C. All purified proteins appeared homogeneous by SDS−PAGE.

Radiolabeling of Proteins. Purified factor XI and prothrombin were radiolabeled with ^{125}I by a minor modification *(24)* of the iodogen method to a specific activity of 5×10^6 cpm/μg of factor XI and 2.5×10^6 cpm/μg for prothrombin. The radiolabeled proteins 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 (Bio-Rad Laboratories, Richmond, CA).

Peptide Synthesis. Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer by a modification of the procedure described by Kent and Clark-Lewis *(28)* and purified to >99% purity using reverse-phase high-performance liquid chromatography (HPLC). The sequences of the synthetic peptides utilized in this study were previously published *(14, 21−23)*. All the peptides utilized in this work were rationally designed, conformationally constrained synthetic peptides based upon previously published *(14, 21−23)* molecular models for the A1, A2, A3, and A4 domains of factor XI. Each peptide was separately modeled using energy minimization calculations *(21)* that confirmed that the modeled peptides assumed a conformation similar to that predicted for the relevant Apple domain. A previously published method *(29)* was used to oxidize the two cysteine residues in each peptide to form a disulfide bond and to conformationally constrain the peptide. The thrombin receptor peptide SFLLRN-amide *(30)* was synthesized using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry on an Applied Biosystems 430A Synthesizer and purified to >99% purity using reverse-phase HPLC.

High-Performance Liquid Chromatography. The HPLC system employed was from Waters (Waters 600 Gradient Module, Model 740 Data Module, Model 46K Universal Injector, and Lambda-Max Model 481 Detector, 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 *(29)*.

Characterization of Synthetic Peptides. All the peptides utilized in this study were examined by HPLC (both reverse phase and gel filtration), and all demonstrated a single homogeneous peak (data not shown). When the 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) or DTNB]. It was determined *(31)* that there was less than 0.02 mol of free SH per mole of peptide, which further verifies that these peptides were homogeneous preparations consisting of intramolecular disulfide-bonded peptide.

Assays of Factor XI Activation. Activation of factor XI (60 nM) by thrombin (1.25 nM) was measured by chromogenic assay. Incubations were carried out in 200 μL of Tris (50 mM), NaCl (150 mM), pH 7.3, with 1% bovine serum albumin and dextran sulfate (1 μg/mL, av M_r = 500 000; Sigma Chemical Co., St. Louis, MO) or gel-filtered platelets at 37 °C. After diluting to a final volume of 1 mL with Tris (50 mM), NaCl (150 mM), pH 7.3, with 1% bovine serum albumin containing 600 μM S-2366 (Glu-Pro−Arg *p*-nitroanilide; Chromogenix, Mölndal, Sweden), the amount of free *p*-nitroaniline was determined by measuring the change in absorbance at 405 nm (A_{405}). The amount of factor XIa generated was assayed by reference to a standard curve constructed using purified factor XIa. Alternatively, the proteolytic activation of factor XI (60 nM) by thrombin (1.25 nM) in the presence of trace quantities of ^{125}I-labeled factor XI was assessed at various time points by SDS−PAGE.

Effect of Peptides on the Rate of Activation of Factor XI by Thrombin. The assay procedure was the same as described above except that thrombin (1.25 nM) was incubated for 5 min at 37 °C with either protein, peptide, or buffer solution before the addition of factor XI (60 nM). In experiments in which platelets were present, platelets were first incubated with $ZnCl_2$ (25 μM), $CaCl_2$ (2 mM), thrombin receptor peptide, SFLLRN-amide (25 μM), and HK (45 nM).

Preparation of Washed Platelets. Platelets were prepared as described *(14)*. Platelet-rich plasma obtained from citrated

human blood was centrifuged, and the platelets were resuspended in calcium-free Hepes−Tyrodes buffer (126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.38 mM NaH_2PO_4, 5.6 mM dextrose, 6.218 mM sodium Hepes, 8.782 mM Hepes free acid, 0.1% BSA), pH 6.5, and gel-filtered on a column of Sepharose 2B equilibrated in calcium-free Hepes−Tyrodes buffer, pH 7.2. Platelets were counted electronically (Coulter Electronics, Hialeah, FL).

Binding of Factor XI to HK. The binding of factor XI to HK was studied using poly(vinyl chloride) microtiter plates, the wells of which were coated with HK by incubation with 100 mL of the protein (100 μg/mL) for 2 h at room temperature. The binding assay and conditions used have been previously described *(20, 21)*. Briefly, after residual binding sites in the wells were blocked, 100 μL of a mixture of ^{125}I-labeled factor XI and either buffer or peptides was added to the wells and incubated for 3−4 h at room temperature. The wells were thoroughly washed with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1.0 mg/mL bovine serum albumin, dried, and counted in a 1470 Wizard Automatic Gamma Counter (Wallac Inc., Gaithersburg, MD).

Binding of Prothrombin to Factor XI or HK. The binding of prothrombin to factor XI or HK was studied using polyvinyl chloride microtiter plates, the wells of which were coated with factor XI or HK 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 ^{125}I-prothrombin (50−1400 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 Wizard Automatic Gamma Counter (Wallac Inc.).

Binding Experiments. Platelets were prewarmed and incubated at a concentration of $(2-4) \times 10^8$/mL in calcium-free Hepes−Tyrodes buffer, pH 7.3, in a 1.5 mL Eppendorf plastic centrifuge tube with a mixture of radiolabeled factor XI, unlabeled factor XI, divalent cations, a thrombin receptor activation peptide (SFLLRN-amide, as a platelet agonist) *(14)*, and HK, or prothrombin, or other proteins. All incubations were performed at 37 °C without stirring after initial mixing of the reaction mixture. At various times after addition of all the components, aliquots were removed (100 μL) and centrifuged through a mixture of silicone oils as described *(14)*. Unless otherwise stated, total binding is shown, uncorrected for any nonsaturable component.

Competition Experiments. Platelets were incubated with various mixtures and concentrations (see figure legends) of $ZnCl_2$, $CaCl_2$, thrombin receptor peptide, SFLLRN-amide, HK or prothrombin, and ^{125}I-factor XI and mixed with samples of various peptides and buffers. After 30 min, samples were centrifuged. Binding of ^{125}I-factor XI was compared to control binding in the absence of proteins or peptides.

Calculation of Binding Constants and Number of Sites. Binding data were analyzed according to the method of Scatchard *(32)*. Individual data points were averages of triplicate determinations. "Nonspecific" binding, apparent dissociation constants, and numbers of binding sites were calculated. Different concentrations of total ligand were made by mixing increasing amounts of unlabeled protein with a constant amount of radiolabeled protein. The nonspecific binding was calculated by assuming that it represented an infinitely nonsaturable component of the total binding *(33)*.

RESULTS

Factor XI Activation by Thrombin in the Presence of Activated Human Platelets. Factor XI activation in the classic intrinsic pathway is facilitated by negatively charged substances such as glass or kaolin *(2)*. Also, a variety of substances will promote thrombin-mediated factor XI activation such as sulfatides, glycosaminoglycans, heparin, dextran sulfate, and dermatan sulfate *(8, 9, 34, 35)*. However, the most effective surface reported so far is dextran sulfate which increases factor XI activation by thrombin 2000-fold *(8, 9)*. Since factor XI binds to activated platelets *(12, 14)* which promote the proteolytic activation of factor XI by factor XIIa *(13, 36)*, we determined whether activated platelets could substitute for dextran sulfate and enhance thrombin-catalyzed factor XI activation. The data presented in Figure 1A demonstrate that in the presence of added HK (45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM), platelets (activated with thrombin receptor activation peptide, SFLLRN-amide) promote the activation of factor XI by thrombin at initial rates ∼5-fold greater than those obtained with dextran sulfate. To confirm the formation of factor XIa, samples of identical incubation mixtures with added ^{125}I-labeled factor XI were examined by SDS gel electrophoresis. A 50 kDa band and a 30 kDa band, corresponding to the heavy and light chains of factor XIa, increased in accordance with an increase in the amidolytic activity (Figure 2). In the presence of activated platelets, rapid (within 2 min) and complete proteolytic cleavage of factor XI was observed, whereas in the absence of platelets, no proteolytic cleavage of factor XI (60 nM) in the presence of thrombin (1.25 nM) was observed during a 4-h time course. When HK was absent from the incubation mixture, the initial rate of factor XI activation in the presence of activated platelets was about 40% of that observed in the presence of HK, and the amount of factor XIa formed was only 25% of that with HK present, suggesting that the preferred substrate for thrombin is factor XI bound to the activated platelets, shown previously *(12)* in the absence of HK to be ∼12−40% of that occurring in the presence of HK. Surprisingly, even when thrombin was excluded from the incubation mixture with platelets, HK, and factor XI, factor XIa generation occurred at rates about one-fifteenth of those with thrombin present, suggesting autoactivation of factor XI (by factor XIa), similar to that observed by other workers in the presence of dextran sulfate *(8, 9)*. When platelets were not activated or were excluded from the incubation mixture or when factor XI was absent, very low rates of factor XI activation (<0.5% of those observed with activated platelets, HK, and factor XI present) were observed.

This comparison of activated platelets and dextran sulfate as a surface for factor XI activation is expanded in Table 1. Here it can be seen that in the absence of added HK, the initial rates of factor XI activation by thrombin are similar in the presence of activated platelets or dextran sulfate. When HK (45 nM) was present, however, there was a 2.6-fold increase in the initial rate of factor XIa formation with platelets, whereas a slight inhibition of factor XI activation by HK (45 nM) occurred with dextran sulfate. The experi-

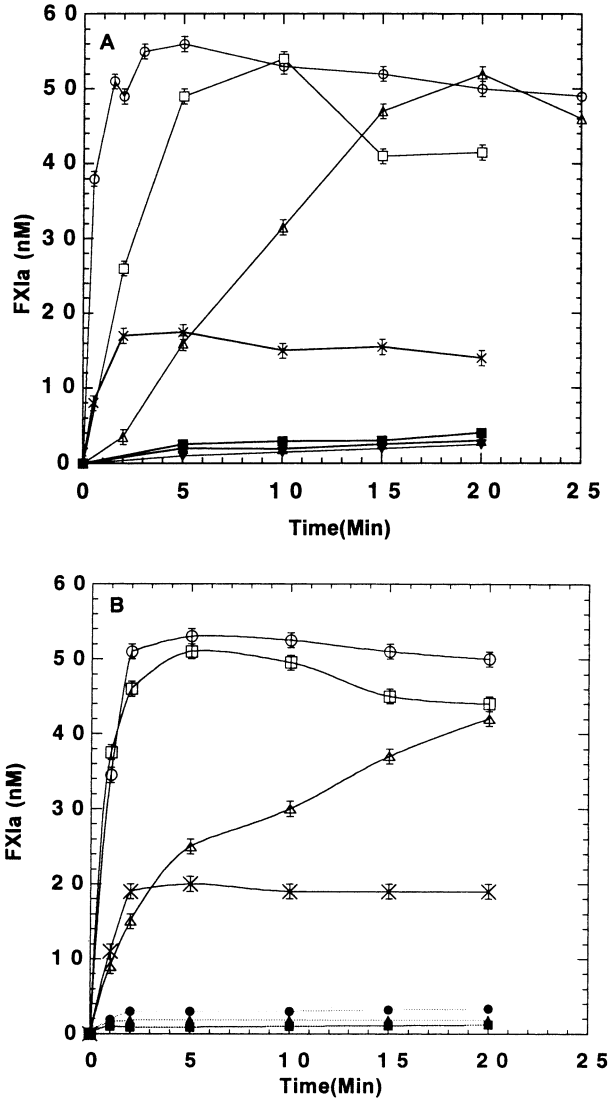

FIGURE 1: Effects of activated platelets or dextran sulfate on the rate of activation of factor XI (60 nM) by thrombin (1.25 mM). Gel-filtered platelets (1×10^8 platelets/mL) activated with the thrombin receptor peptide (SFLLRN-amide, 25 μM) or dextran sulfate (1 μg/mL) were incubated with $ZnCl_2$ (25 μM), $CaCl_2$ (2 mM), and either HK (45 nM) or prothrombin (1.2 μM) in the presence or absence of factor XI (60 nM), for 5 min at room temperature after which thrombin (1.25 nM) was added and the mixture was further incubated at 37 °C. The rate of factor XIa formation was determined as described under Experimental Procedures. Panel A: Data shown are mean values ± SEM ($n = 4$) of measurements of factor XIa with activated platelets, factor XI, HK, and thrombin (○); factor XI, HK, thrombin, and dextran sulfate (□); factor XI, HK, and activated platelets in the absence of thrombin (△); factor XI, HK, and unactivated platelets in the absence of thrombin (■); thrombin, HK, and activated platelets in the absence of factor XI (▼); factor XI, HK, and thrombin (▲); or activated platelets in the absence of HK in the presence of factor XI and thrombin (×). Panel B: Data shown are mean values ± SEM ($n = 4$) with activated platelets, prothrombin, $CaCl_2$, factor XI, and thrombin (○); activated platelets, HK, $ZnCl_2$, $CaCl_2$, factor XI, and thrombin (□); activated platelets, prothrombin, $CaCl_2$, and factor XI in the absence of thrombin (△); activated platelets, factor XI, and thrombin in the absence of prothrombin and HK (×); unactivated platelets, prothrombin, $CaCl_2$, and factor XI in the absence of thrombin (■); factor XI and thrombin (▲); and activated platelets, prothrombin, $CaCl_2$, and thrombin in the absence of factor XI (●).

ments, shown in Table 1 and Figure 1, were carried out in the presence of HK (45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM) since these conditions have been shown to be optimal for binding of factor XI to the platelet surface *(12)*. At higher concentrations of HK up to the physiological concentration in plasma (636 nM), there was a concentration-dependent decrease in the rates of factor XI activation in the presence

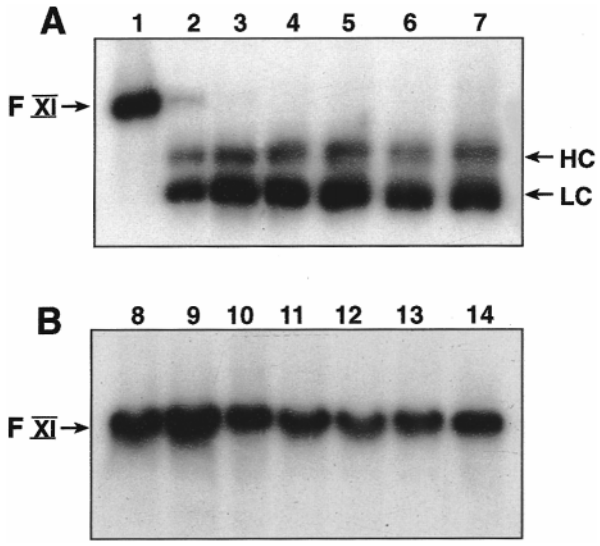

FIGURE 2: Effect of platelets (1×10^8 platelets/mL, activated with SFLLRN-amide, 25 μM) on the activation of ^{125}I-factor XI (60 nM) by thrombin (1.25 nM) in the presence of $ZnCl_2$ (25 μM), $CaCl_2$ (2 mM), and HK (45 nM). ^{125}I-Factor XI is cleaved to heavy chain (HC) (50 000 Da) and light chain (LC) (30 000 Da) in the presence of activated platelets (A) but remains as a single chain (80 000 Da) in the absence of platelets (B). Autoradiographs are shown of samples that were reduced with 5% β-mercaptoethanol prior to SDS−PAGE in 12% acrylamide. Platelets present: lanes 1, 2, 3, 4, 5, 6, and 7 are samples analyzed at 0, 1, 2.5, 3.5, 5, 10, and 15 min, respectively, of an incubation of ^{125}I-factor XI (60 nM) and platelets (1×10^8 platelets/mL) and thrombin (1.25 nM). Platelets absent: lanes 8, 9, 10, 11, 12, 13, and 14 are samples analyzed at 0, 5, 10, and 20 min and 1, 2, and 4 h, respectively.

Table 1: Effects of HK and Prothrombin on the Initial Rates of Factor XI Activation by Thrombin in the Presence of Activated Platelets or Dextran Sulfate[a]

surface	HK (nM)	prothrombin (μM)	initial rate of factor XIa formation (nM/min)[b]
activated platelets	0	−	9.0 (±1.0)
	45	−	23.5 (±2.0)
	90	−	15.45 (±1.75)
	270	−	14.0 (±1.5)
	636	−	9.1 (±1.0)
	636	1	25.0 (±2.0)
	636	3	24.85 (±1.95)
	636	(PF1.2) 1	24.75 (±2.10)
	636	(PF1) 1	9.5 (±1.0)
dextran sulfate	0	−	12.5 (±1.2)
	45	−	11.0 (±1.45)
	90	−	7.0 (±0.5)
	270	−	5.5 (±0.75)
	636	−	5.0 (±0.5)
	636	1	12.4 (±1.5)
	636	3	12.5 (±1.25)
	636	(PF1.2) 1	12.5 (±1.70)
	636	(PF1) 1	5.3 (±0.60)

[a] The Experiment Was Performed as Described in the Legend to Figure 1 with the exception of the Addition to the Incubation Mixtures of HK and/Or Prothrombin at the Concentrations Indicated. the Rate of Factor XI Formation Was Determined as Described under Experimental Procedures. [b] Data shown represent the means ± standard deviation of three observations.

of either activated platelets or dextran sulfate. This inhibition by HK of factor XIa generation was completely reversed in the presence of prothrombin at physiological concentrations (1−3 μM), such that initial rates of factor XIa formation in the presence of physiological concentrations of HK (636 nM), prothrombin (1−3 μM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM) and activated platelets were about twice those observed under the same conditions with dextran sulfate replacing platelets. By comparison, negligible initial rates of factor XI activation were observed in the absence of activated platelets or dextran sulfate either in the presence or in the absence of thrombin or with unactivated platelets without thrombin or in the absence of factor XI, so that the rate enhancement of thrombin-catalyzed factor XI activation observed due to the presence of activated platelets was well in excess of 250-fold (Figure 1A).

Effect of Prothrombin, PPACK-Treated Thrombin, PF1, and PF1.2 on the Binding of ^{125}I-Factor XI to HK. Previously we defined a binding site for HK in the A1 domain of factor XI *(20, 21)*. Since thrombin can activate factor XI and HK inhibits the activation of factor XI by thrombin, we have also identified a thrombin binding site in the A1 domain of factor XI that is contiguous with but separate and distinct from the HK binding site *(19)*. Therefore, we determined the effects of prothrombin, active-site-inhibited (PPACK-treated) thrombin, PF1, and PF1.2 on the binding of factor XI to HK.

The experiment presented in Figure 3 demonstrates that prothrombin inhibits the binding of ^{125}I-factor XI to HK ($IC_{50} = 2 \times 10^{-6}$ M) as does PPACK-treated thrombin ($IC_{50} = 1 \times 10^{-5}$ M). Surprisingly, PF1.2 inhibited the binding of factor XI to HK (with an $IC_{50} = 3.5 \times 10^{-7}$ M) whereas PF1 had no effect. Moreover, when PF1.2 and PPACK-treated thrombin were added together at equimolar concentrations, their effects on factor XI binding to HK were additive and similar to that of prothrombin ($IC_{50} = 2 \times 10^{-6}$ M). These experiments suggest that prothrombin, as well as thrombin, binds to factor XI to a site spatially contiguous with the HK binding site and that PF1.2 (possibly utilizing the Kringle II domain) may also bind factor XI near this site, thereby displacing HK from the A1 domain of factor XI. Consistent with this conclusion is the observation that PF1.2 (but not PF1), as well as prothrombin, reverses the inhibitory effect of HK on thrombin-catalyzed factor XI activation in the presence of either dextran sulfate or platelets (Table 1).

Interaction of Prothrombin with Factor XI and HK. Since prothrombin, PPACK-thrombin, and PF1.2 inhibit the binding of factor XI to HK (Figure 3), we determined whether prothrombin binds directly to factor XI or to HK. Therefore, we carried out experiments in which either factor XI or HK was bound to the wells of a microtiter plate to determine whether prothrombin could bind to either of these proteins. Our results indicate that ^{125}I-prothrombin binds factor XI in a saturable manner with a $K_d \sim 250$ nM whereas only a low level of nonspecific, nonsaturable binding of ^{125}I-prothrombin was demonstrable, indicating no evidence of saturable, specific binding of prothrombin to HK (data not shown).

Effect of Synthetic Peptides on the Activation of Factor XI by Thrombin in the Presence of Activated Platelets or Dextran Sulfate. We have previously identified amino acid sequences in the Apple domains within the heavy-chain region of factor XI that interact with HK, thrombin, factor IX, platelets, and factor XIIa *(14, 19−23)*. Since both activated platelets and dextran sulfate enhance thrombin-catalyzed factor XI activation, we determined which binding domains are important in facilitating these effects. The data presented in Table 2 demonstrate the effects of various conformationally constrained synthetic peptides on the activation of factor XI by thrombin in the presence of activated platelets or dextran sulfate. The A3 domain peptide Asn 235−Arg 266 (the platelet binding site, see ref *14)* inhibited the activation of factor XI by thrombin in the presence of activated platelets with an $IC_{50} = 0.5 \times 10^{-5}$ M, whereas in contrast this peptide had no inhibitory effect at concentrations as high as 10^{-3} M in the presence of dextran sulfate. The A1 domain synthetic peptide Ala 45−Arg 54 (the thrombin binding site in factor XI, see ref *19*) also inhibited thrombin-catalyzed factor XI activation in the presence of either activated platelets ($IC_{50} = 0.5 \times 10^{-5}$ M) or dextran sulfate ($IC_{50} = 2 \times 10^{-5}$ M). In contrast, the A1 domain peptide Phe 56−Ser 86 (comprising the HK binding site) *(20, 21)* while inhibiting thrombin-catalyzed factor XI activation on dextran sulfate ($IC_{50} = 10^{-4}$ M) had no inhibitory effect in the presence of activated platelets. Synthetic peptides representing sequences in the A2 domain (Ala 134−Ala 176, containing a substrate binding site for factor IX) *(22)* and A4 domain (Gly 326−Lys 356, a factor XIIa binding site) *(23)* were unable to inhibit the activation of factor XI by thrombin either with platelets or with dextran

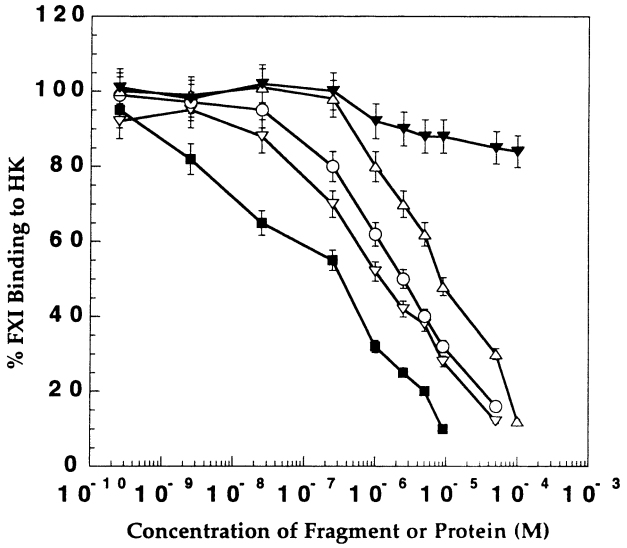

FIGURE 3: Effects of prothrombin and its fragments on the binding of ^{125}I-factor XI to HK. ^{125}I-Factor XI (25 nM) was incubated with prothrombin or its fragments at the concentrations indicated. Binding of ^{125}I-labeled factor XI to immobilized HK was determined as described under Experimental Procedures. Data points (mean ± SEM of 3 determinations) are shown for prothrombin (○); PPACK-treated thrombin (△); prothrombin fragment 1.2 (■); prothrombin fragment 1 (▼); or equimolar concentrations of prothrombin fragment 1.2 and PPACK-treated thrombin (▽). When HK was not bound to the wells of the microtiter platelets, the amount of ^{125}I-labeled factor XI was <2% of the control value, and the maximum variation of cpm bound for each observation was <2% of the total cpm bound. One hundred percent binding of factor XI represents an average of 10 552 cpm bound, whereas 0% binding of factor XI represents 0 cpm bound after subtracting an average of 159 cpm, representing the negative control in which ^{125}I-labeled factor XI was added to wells coated with bovine serum albumin instead of HK.

Table 2: Effects of Synthetic Peptides on Thrombin-Catalyzed Activation of Factor XI in the Presence of Activated Platelets or Dextran Sulfate[a]

surface	peptides (domain)	ligand[b]	reference[c]	IC_{50} (M)
activated platelets	Ala 45−Arg 54 (A1)	thrombin	*19*	0.5×10^{-5} M
	Phe 56−Ser 86 (A1)	HK	*20, 21*	NE[d]
	Ala 134−Ala 176 (A2)	factor IX	*22*	NE[d]
	Asn 235−Arg 266 (A3)	platelets	*14*	0.5×10^{-5} M
	Ala 317−Gly 350 (A4)	factor XIIa	*23*	NE[d]
dextran sulfate	Ala 45−Arg 54 (A1)	thrombin	*19*	2×10^{-5} M
	Phe 56−Ser 86 (A1)	HK	*20, 21*	1×10^{-4} M
	Ala 134−Ala 176 (A2)	factor IX	*22*	NE[d]
	Asn 235−Arg 266 (A3)	platelets	*14*	NE[d]
	Ala 317−Gly 350 (A4)	factor XIIa	*23*	NE[d]

[a] The experiment was performed as described in the legend to Figure 1 except that peptides were added to the incubation mixtures at concentrations ranging from 10^{-8} to 10^{-3} M. IC_{50} is defined as the concentration (M) of peptide required to inhibit the rate of thrombin-catalyzed activation of factor XI by 50%. [b] Shown in this column are the ligands previously shown to bind to the Apple domain sequences indicated. [c] Shown in this column are the numbers of references given in the Reference list that present data on the identification and characterization of the ligand binding domain indicated. [d] NE = no inhibitory effect observed at concentrations up to 10^{-3} M.

sulfate as a surface (Table 2). This experiment reveals that a binding site for thrombin in the A1 domain (Ala 45−Arg 54) and a platelet receptor binding site in the A3 domain (Asn 235−Arg 266) are important amino acid sequences involved in thrombin-catalyzed factor XI activation in the presence of activated platelets. In contrast, the mechanism of thrombin-catalyzed factor XI activation appears to be different on dextran sulfate since the A1 domain HK binding site (Phe 56−Ser 86) is essential for the dextran sulfate-mediated but not the platelet-mediated reaction, whereas the A3 domain platelet binding site is essential for the reaction on the platelet surface but not on dextran sulfate.

Prothrombin Can Promote the Binding of Factor XI to Activated Human Platelets. The data presented in Figure 1 and Table 1 demonstrate that platelets, like dextran sulfate, can promote the activation of factor XI by thrombin and that prothrombin (at physiological concentrations, i.e., 1−3 μM) can reverse the inhibitory effect of HK (at physiological concentrations, i.e., ~640 nM) on thrombin-catalyzed factor XI activation in the presence of either activated platelets or dextran sulfate. Since HK promotes the specific, high-affinity binding of factor XI to the surface of stimulated human platelets in the presence of zinc ions and calcium ions *(12)* and factor XI binding to platelets promotes the proteolytic activation of factor XI by factor XIIa *(13)*, we postulated that thrombin-mediated factor XI activation might also require factor XI binding to activated platelets. Since (1) both prothrombin and PF1.2 inhibit the binding of factor XI to HK (Figure 1), (2) prothrombin binds to factor XI with a K_d ~250 nM (Figure 3), and (3) prothrombin binds in a saturable and reversible manner to the activated platelet surface *(37)*, we further postulated that complex formation between factor XI and prothrombin can promote the binding of factor XI to the platelet surface in the absence of HK, since this would explain the capacity of prothrombin to promote optimal rates of factor XI activation by thrombin in the presence of inhibitory concentrations of HK. Figure 4A demonstrates that either prothrombin (3 μM) or HK (42 nM), in the presence of $ZnCl_2$ (25 μM) and $CaCl_2$ (2 mM), can promote the binding of ^{125}I-factor XI to approximately the same number of sites on the activated platelet surface. This binding is not observed in the absence of either prothrombin or HK or when prothrombin is present but the divalent metal ions (Ca^{2+} and Zn^{2+}) are absent. The amount of factor XI binding is a linear function of prothrombin concentration below those present in plasma (1−3 μM) and is saturable at prothrombin concentrations of 3−6 μM (Figure 4A, inset). Binding to stimulated platelets in the presence of either HK (45 nM) or prothrombin (3 μM), in the presence of $CaCl_2$ (2 mM) or $ZnCl_2$ (25 μM), reached a plateau in ~20 min. Since omission of prothrombin from the incubation mixture also decreases binding to the background level, it may be inferred that prothrombin, like HK *(12)*, can act as a cofactor in the interaction of factor XI with stimulated platelets and that the binding of factor XI to activated platelets is quantitatively similar when either HK or prothrombin is used as a cofactor.

Effect of Calcium and Zinc Ions on the Ability of Prothrombin To Promote Binding of Factor XI to Activated Platelets. Binding of factor XI to platelets in the presence of the cofactor, HK, requires the presence of zinc ions and is potentiated by calcium ions *(12)*. Optimal concentrations of 25 μM $ZnCl_2$ and 2 mM $CaCl_2$ have been determined using HK as the cofactor *(12)*. Specific binding of HK to the platelet surface also requires the presence of zinc ions *(38)*. We therefore determined the divalent cation requirements using prothrombin as a cofactor for binding of factor XI to the platelet surface. The data (Figure 4B) show that prothrombin (3 μM) can promote the binding of factor XI to activated platelets in the presence of $CaCl_2$ (2 mM), whereas $ZnCl_2$ (25 μM) was neither necessary nor sufficient to promote factor XI binding to activated platelets in the presence of prothrombin.

Reversibility and Specificity of Factor XI Binding. We next carried out experiments to examine the capacity of unlabeled factor XI (at 240-fold excess) to displace ^{125}I-labeled factor XI from the platelet surface in the presence of prothrombin and Ca^{2+} ions (Figure 4B, inset). Unlabeled factor XI added at 15 min resulted in dissociation of bound radiolabeled factor XI, with 30−40% being removed during the first minute followed by a slower dissociation of >95% of bound ligand. When the addition was made at 40 min, a slower rate of dissociation was observed with a subsequent plateau at ~70% dissociation. Prekallikrein, added at 240-fold molar excess, was unable to dissociate or prevent factor XI binding in the presence of prothrombin (3 μM) and $CaCl_2$ (2 mM).

Determination of Stoichiometry and Affinity of Factor XI Binding. The foregoing data indicate that factor XI binding to platelets is specific and >95% reversible and is thus

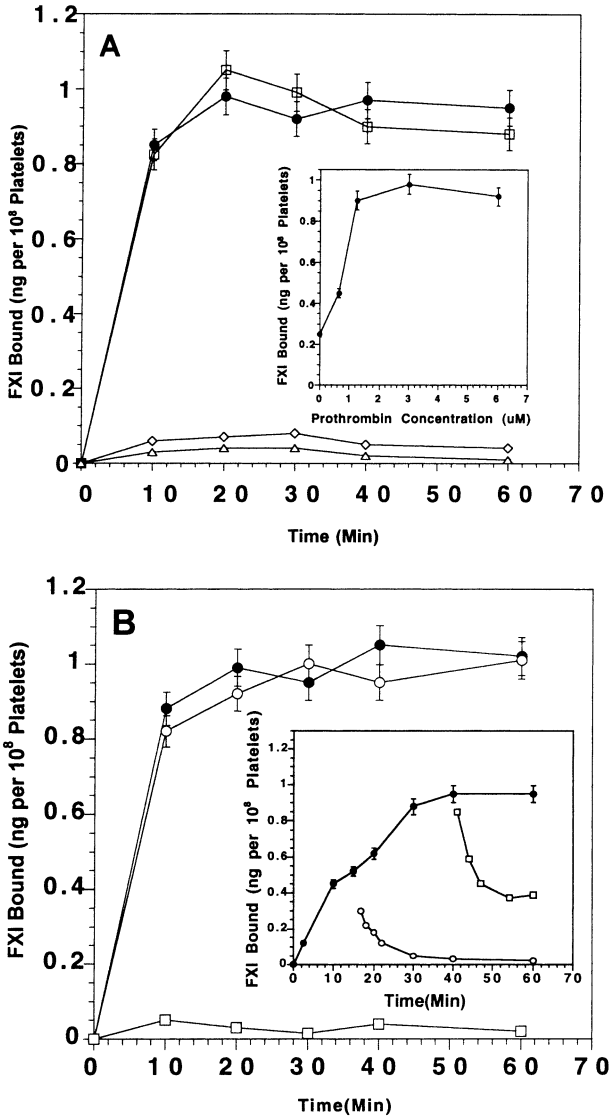

FIGURE 4: The binding of ^{125}I-factor XI to platelets in the presence of HK or prothrombin. Gel-filtered platelets (4.2×10^8 platelets/mL) were incubated after initial mixing but without constant stirring at 37 °C with the thrombin receptor peptide, SFLLRN-amide (25 μM), ^{125}I-factor XI (25 ng/mL), and with various concentrations of HK or prothrombin as indicated below. At the times indicated, aliquots were removed and centrifuged as described under Experimental Procedures. Each data point is an average of triplicate determinations. The maximum variation of cpm bound for each experimental observation was <2% of the total cpm bound. When ^{125}I-factor XI was incubated with platelets at 0 time, the amount of factor XI bound was <1% of the maximum amount at 30 min. Panel A: HK (45 nM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM) (●); prothrombin (3 μM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM) (□); $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM), in the absence of HK and prothrombin (◇); and prothrombin (3 μM), in the absence of $CaCl_2$ and $ZnCl_2$ (△). The inset shows the amount of factor XI bound at 30 min of incubation (37 °C) under the same experimental conditions with ^{125}I-factor XI, gel-filtered platelets, $ZnCl_2$, $CaCl_2$, thrombin receptor peptide (SFLLRN-amide), and prothrombin at the concentrations shown. Panel B: Prothrombin (3 μM), $CaCl_2$ (2 mM), $ZnCl_2$ (25 μM) (●); prothrombin (3 μM) and $CaCl_2$ (2 mM) in the absence of $ZnCl_2$ (○); prothrombin (3 μM) and $ZnCl_2$ (25 μM) in the absence of $CaCl_2$ (□). Inset: Reversibility of ^{125}I-factor XI binding to platelets in the presence of prothrombin and $CaCl_2$. The time course of ^{125}I-factor XI (25 ng/mL) binding to stimulated platelets was determined at 37 °C in the presence of 2.0 mM $CaCl_2$ (●). At 15 and 40 min intervals, 240 molar excess unlabeled factor XI was added to the incubation mixture, and the binding of ^{125}I-factor XI was measured (○; □).

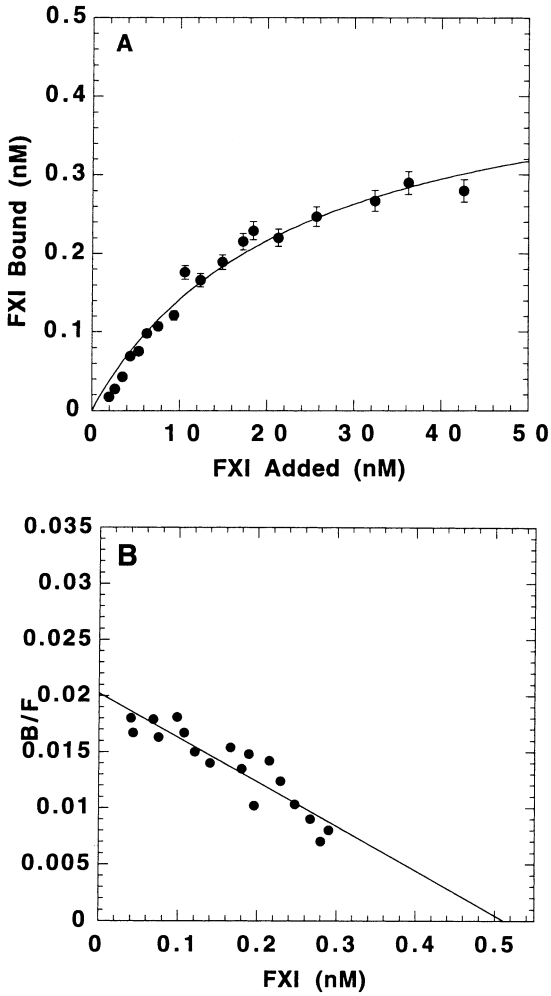

FIGURE 5: Saturable, specific binding of ^{125}I-factor XI to platelets in the presence of prothrombin. Platelets were incubated at 37 °C with $CaCl_2$ (2 mM), thrombin receptor peptide SFLLRN-amide (25 μM), PT (3 μM), and mixtures of ^{125}I-factor XI and unlabeled factor XI at various concentrations. Binding was determined at 30 min. (A) Amount of ^{125}I-factor XI bound at different input concentrations; specific binding (●) is shown after subtracting the amount bound in the presence of a large molar excess of unlabeled factor XI. (B) Scatchard analysis of the data shown in (A) The line is a best fit of data from triplicate samples. The apparent dissociation constant and number of binding sites were calculated as parameters.

amenable to Scatchard analysis. Binding of various concentrations of factor XI to stimulated platelets was determined in the presence of 3 μM prothrombin and 2 mM $CaCl_2$. Saturable binding of ^{125}I-factor XI to stimulated platelets was observed (Figure 5A). Nonspecific binding, as calculated from competition of unlabeled factor XI, was subtracted at each data point. The results demonstrate that saturable binding was achieved at factor XI concentrations above those present in plasma (25 nM). When the saturation data were analyzed by the method of Scatchard *(32)*, an apparent dissociation constant ($K_{d_{app}}$) of 25 ± 2.5 nM and a total of 947 ± 150 binding sites were calculated (Figure 5B).

Effect of the Apple 3 Domain Peptide on Prothrombin-Mediated Binding of Factor XI to Activated Platelets. The results presented above indicate that activated platelets enhance thrombin-catalyzed factor XI activation in the presence of HK (Figure 1) and that the A3 domain peptide, Asn 235−Arg 266 (an analogue of the platelet binding site), inhibits the activation of factor XI by thrombin in the

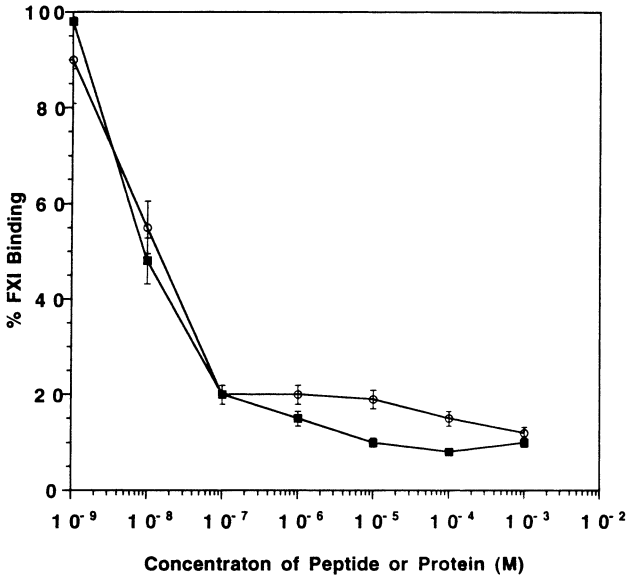

FIGURE 6: Effect of the Apple 3 domain peptide, Asn 235−Arg 266, on prothrombin-mediated binding of ^{125}I-factor XI to platelets. ^{125}I-Factor XI (25 ng/mL), gel-filtered platelets (4.2×10^8 platelets/mL), prothrombin (1.2 μM), $CaCl_2$ (2 mM), and thrombin receptor peptide, SFLLRN-amide (25 μM), were incubated for 30 min at 37 °C either with the conformationally constrained synthetic peptide (Asn 235−Arg 266, ○) or with unlabeled factor XI (■) at various concentrations or with buffer solution. Aliquots were removed, and separation of bound from free ligand was accomplished as described under Experimental Procedures. Each data point is an average of triplicate determinations. When ^{125}I-factor XI was incubated with platelets at 0 time, the amount of ^{125}I-factor XI bound was <1% of the control value (incubated for 30 min), and the maximum variation of cpm bound for each experimental observation was <2% of the total cpm bound. One hundred percent binding of factor XI represents an average of 90 590 cpm bound whereas 0% binding of factor XI represents 0 cpm bound after subtracting an average of 150 cpm representing the negative control in which ^{125}I-factor XI was incubated with platelets at 0 time.

presence of HK and activated platelets (Table 2). Since this peptide also inhibits factor XI binding to platelets in the presence of HK (45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM) with an IC_{50} ~10 nM *(14)*, we examined this peptide for its ability to inhibit factor XI binding to platelets in the presence of the cofactor prothrombin and calcium ions. The results (Figure 6) show that the A3 peptide (Asn 235−Arg 266) inhibits the binding of factor XI to platelets with an IC_{50} ~10 nM, which is almost identical with that observed with unlabeled factor XI. This result confirms the conclusion that a binding site for platelets in the A3 domain of factor XI is exposed in the presence of prothrombin and Ca^{2+} ions.

Factor XI Activation by Thrombin in the Presence of Prothrombin and Activated Human Platelets. Since activated platelets promote thrombin-catalyzed factor XI activation under conditions shown to be optimal for binding of factor XI to the platelet surface *(12)*, i.e., in the presence of HK (45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM) (Figure 1A), and since prothrombin is a cofactor that can substitute for HK in the binding of factor XI to platelets (Figures 4 and 5), we next examined the effect of prothrombin on thrombin-catalyzed factor XI activation. The data in Figure 1B demonstrate that in the presence of prothrombin (1.2 μM) and $CaCl_2$ (2 mM), platelets (activated with thrombin receptor peptide SFLLRN-amide) promote the activation of factor XI by thrombin at rates similar to those obtained with HK (45 nM) in the presence of $ZnCl_2$ (25 μM). When HK and prothrombin were absent from the incubation mixture, the initial rate of factor XI activation in the presence of activated platelets was ~40% of that observed in the presence of HK or prothrombin, and the amount of factor XIa formed was only 25% of that with HK or prothrombin present, suggesting that the preferred substrate for thrombin is factor XI bound to activated platelets. By comparison, negligible initial rates of factor XI activation were observed in the absence of activated platelets, either in the presence or in the absence of thrombin, or in the absence of factor XI, so that the rate enhancement of thrombin-catalyzed factor XI activation due to the presence of activated platelets in the presence of either HK or prothrombin was well in excess of 250-fold. The activation of factor XI observed in the absence of thrombin with activated platelets (Figure 1B) could represent autoactivation as described previously *(8, 9)*. We conclude that the binding of factor XI to the platelet surface in the presence of either HK or prothrombin is required for the marked enhancement of factor XI activation by thrombin observed in the presence of activated platelets.

DISCUSSION

The mechanisms and physiological importance of thrombin-catalyzed factor XI activation have been the focus of intensive study *(8, 9, 15, 16, 19, 34, 35)* since this pathway of activation of intrinsic coagulation could explain the absence of hemostatic deficiency in patients with "contact factor" deficiencies. Although factor XI is activated by thrombin in a purified system with dextran sulfate, it has been suggested that this reaction may not proceed as readily in plasma, since although HK promotes the factor XIIa-mediated reaction, it inhibits thrombin-catalyzed activation of factor XI *(8, 9, 15, 16)*. Conflicting reports provide evidence that activation of factor XI by thrombin proceeds *(8, 9, 34, 35)* or does not proceed *(15, 16)* in plasma. Von dem Borne and co-workers determined that protection of a clot from fibrinolytic attack was dependent on the thrombin that was generated in a factor XI-dependent manner via the intrinsic pathway *(17)*. Also, it was shown that thrombin-mediated factor XI activation could take place in the absence of an activating surface *(18)*. However, large quantities of thrombin (20 nM or ~2 units/mL) were necessary for this to occur *(18)*. Previously, it was shown that thrombin (1.25 nM) cannot activate plasma levels of factor XI (60 nM) unless a surface such as dextran sulfate is present *(8, 9)*. We were, therefore, interested in addressing the following questions: Is thrombin a physiologically relevant activator of factor XI at concentrations of reactants present in plasma? What is the mechanism by which HK can inhibit thrombin-catalyzed factor XI activation? What are the physiologically relevant cell surfaces involved in thrombin-catalyzed factor XI activation?

Our present studies support the conclusion that activated platelets can promote thrombin-catalyzed factor XI activation at initial rates 2−5-fold greater than those obtained in the presence of dextran sulfate (Figure 1A, Figure 2, and Table 1). Although our observations are consistent with the conclusion that thrombin is directly responsible for the enhanced proteolytic activation of factor XI, it is possible that the thrombin may be providing additional stimulation

to the platelets which are elaborating some factor that activates factor XI. It is clear, however, that the dramatic enhancement by activated platelets of factor XIa generation rates measured by amidolytic activity truly represents factor XIa and not some other enzyme with chromogenic activity, since proteolytic cleavage of factor XI (Figure 2) correlates very well with generation of factor XIa activity (Figure 1A). These experiments were carried out under conditions previously shown to be optimal for factor XI binding to platelets *(12)*, i.e., in the presence of HK (45 nM), $ZnCl_2$ (25 μM), and $CaCl_2$ (2 mM). The presence of HK at this concentration (45 nM, i.e., ~7% of the plasma concentration), while increasing (~2.5-fold) the initial rates of factor XI activation by thrombin in the presence of activated platelets, had a slight inhibitory effect in the presence of dextran sulfate (Table 1). A concentration-dependent inhibitory effect of HK (45−636 nM) was observed with both dextran sulfate and platelets (Table 1) which we attribute to the capacity of HK to displace thrombin from the A1 domain of factor XI *(19)*. Surprisingly, prothrombin at its normal plasma concentration (1−3 μM) completely reversed this inhibitory effect of HK (Table 1) at its normal plasma concentration (636 nM). This observation led us to postulate that prothrombin might bind either to factor XI or to HK and thereby obviate this inhibitory effect of HK.

Recently, we have identified a sequence of amino acids in factor XI that binds thrombin and is contiguous with the HK binding site *(19)*. Since the precursor of thrombin in plasma is prothrombin, we initially studied the role of PF1 and PF1.2 and prothrombin in their interaction with factor XI and HK. Our experiments support the conclusion that prothrombin as well as thrombin *(19)* binds factor XI at a site that interferes with HK binding to the A1 domain (Phe 56−Ser 86). Moreover, the Kringle II domain of prothrombin also appears to bind to a site contiguous with the HK binding site in the A1 domain. The evidence supporting these conclusions includes the following: (1) ^{125}I-prothrombin binds factor XI in a saturable manner with a K_d ~2.5×10^{-7} M, whereas we observed no saturable specific binding of prothrombin to HK; (2) prothrombin ($IC_{50} = 2 \times 10^{-6}$ M), active-site-inhibited (PPACK-treated) thrombin ($IC_{50} = 10^{-5}$ M), and PF1.2 ($IC_{50} = 3.5 \times 10^{-7}$ M) all inhibit factor XI binding to HK whereas PF1 has no such effect (Figure 3). Since the sole difference between PF1 and PF1.2 is the presence of the Kringle II domain in PF1.2, it follows that the Kringle II domain of prothrombin binds to a site in factor XI that, although not yet mapped in detail, cannot be the same as the site to which thrombin (or active-site-inhibited thrombin) binds since the inhibitory effects of PF1.2 and PPACK-thrombin on factor XI binding to HK are additive and are equal to the effect of intact prothrombin (Figure 3). Furthermore, PF1.2 abrogates the inhibitory effect of HK on thrombin-catalyzed factor XI activation in the presence of dextran sulfate or activated platelets (Table 1), indicating that it cannot bind to the same site on factor XI as does thrombin.

Our previously published results predicted that if HK is bound to the A1 domain, thrombin-mediated activation of factor XI would be blocked and factor XIIa-mediated activation of factor XI would be favored *(19)*. Our present results suggest that prothrombin can utilize two separate and distinct domains, one in the Kringle II domain, the other in the catalytic domain, both of which bind to factor XI and displace HK. It is well-known that factor XI circulates in plasma in an equimolar complex with HK *(11)*. Therefore, the next question to address was whether prothrombin or its fragments might reverse the inhibitory effect of HK on factor XI activation by thrombin when the reaction is carried out on a surface. Our results (Table 1) show that both prothrombin and PF1.2 reverse the inhibition by HK of thrombin-catalyzed factor XI activation in the presence of either dextran sulfate or activated platelets when all reactants are present at concentrations close to those in plasma. Therefore, it seems reasonable to conclude that, at the physiological concentrations of prothrombin and HK in plasma, factor XI activation by thrombin might occur since the inhibitory effects of HK would be obviated in the presence of prothrombin.

We have previously reported evidence of the presence of a specific binding site for platelets in the A3 domain of factor XI *(14)*. Since activated platelets promote the activation of factor XI by thrombin (Figure 1), we then determined that the A3 domain, by interacting with the platelet surface, mediates factor XI binding to activated platelets and facilitates the enhancement by platelets of thrombin-catalyzed factor XI activation (Table 2). The mechanism by which factor XI interaction with its platelet receptor might accelerate its activation by thrombin is unknown but might involve a change in the conformation of factor XI to render it a more favorable substrate for thrombin or might result from colocalization of enzyme and substrate on the platelet surface. Either of these two interpretations is consistent with the results of the experiments which indicate that the acceleration of thrombin-catalyzed factor XI activation is enhanced when HK is present to facilitate the binding of factor XI to the platelet surface (Table 1) and inhibited when factor XI binding to platelets is inhibited by the A3 domain peptide (Table 2). Since the A3 domain peptide, Asn 235−Arg 266, does not inhibit the enhancement of thrombin-catalyzed factor XI activation in the presence of dextran sulfate (Table 2), the mechanism by which activated platelets promote this effect (i.e., through platelet receptor interactions with the A3 domain of factor XI) appears to be different from the mechanism by which dextran sulfate accelerates factor XI activation by thrombin. The conformationally constrained synthetic peptide Phe 56−Ser 86, which inhibits factor XI binding to HK, prevents factor XI activation by thrombin in the presence of dextran sulfate but not in the presence of activated platelets (Table 2). These results suggest that factor XI binds to dextran sulfate through HK where it can be activated by thrombin. The A1 peptide, Ala 45−Arg 54, which comprises the thrombin binding site *(19)*, also inhibited thrombin-catalyzed factor XI activation in the presence of either dextran sulfate or activated platelets as expected (Table 2). It can be concluded that the mechanisms by which activated platelets and dextran sulfate promote thrombin-catalyzed factor XI activation, while generally similar, are different in detail since the binding of factor XI to platelets occurs through the A3 domain (Asn 235−Arg 266) whereas the binding to dextran sulfate apparently is mediated by HK through the A1 domain (Phe 56−Ser 86).

Since prothrombin and PF1.2 but not PF1 bind to factor XI, displace HK from the A1 domain (Figure 3), and obviate the inhibitory effect of HK on thrombin-catalyzed factor XI

activation on the platelet surface (Figure 1A and Table 1), we were led to postulate that complex formation between factor XI and prothrombin can promote the binding of factor XI to the platelet surface in the absence of HK. The results of our studies demonstrate that prothrombin (in the presence of Ca^{2+} ions) can substitute for HK (and Zn^{2+}) as a cofactor for factor XI binding to activated platelets and promote factor XI activation by thrombin on the platelet surface. The evidence supporting this conclusion is as follows: (1) Prothrombin at physiological concentrations (1−3 μM) acts as a cofactor that promotes factor XI binding to the platelet surface (Figure 4A). (2) The binding of factor XI to activated platelets is quantitatively similar when either HK or prothrombin is present as a cofactor (Figure 4A). (3) Prothrombin can promote the binding of factor XI to activated platelets in the presence of 2 mM $CaCl_2$ but not in the presence of $ZnCl_2$ (Figure 4B). In contrast, factor XI binding to platelets mediated by HK requires the presence of $ZnCl_2$ *(12)*. (4) The binding of factor XI to 947 ± 150 sites per activated platelet in the presence of prothrombin is specific, saturable, and reversible with a $K_{d_{app}}$ of 25 ± 2.5 nM (Figure 5). (5) The binding of factor XI to the platelet surface mediated by prothrombin can promote factor XI activation by thrombin on the platelet surface (Figure 1B). (6) The binding of factor XI to activated platelets in the presence of prothrombin and $CaCl_2$ is mediated by a direct interaction of factor XI with the platelet surface since the A3 domain peptide (Asn 235−Arg 266) comprising the platelet binding site in the presence of HK *(14)* inhibits factor XI binding to platelets in the presence of prothrombin (Figure 6).

The conclusions drawn from our present and previously published studies are summarized in schematic form in Figure 7, in which two parallel mechanisms for factor XI binding to the activated platelet surface are represented. Factor XI can bind to the surface of activated platelets (∼1000−1500 sites per platelets) with a K_d of ∼10−20 nM, i.e., at a concentration below that present in normal human plasma (∼25 nM). This interaction is mediated by a protein surface exposed within the A3 domain of factor XI (Asn 235−Arg 266) only after factor XI interacts with a cofactor, either HK (in the presence of Zn^{2+} ions) or prothrombin (in the presence of Ca^{2+} ions). Thus, initially factor XI interacts through its A1 domain either with prothrombin (through its Kringle II domain) or with HK. The fact that the affinity of the factor XI/HK interaction (K_d ∼10^{-8} M) is much greater than that of the factor XI/prothrombin interaction (K_d ∼2.5 × 10^{-7} M) explains the observation *(11)* that factor XI circulates in plasma in complex with HK (concentration ∼640 nM), not prothrombin (concentration 1.5 μM). However, when prothrombin binds to the surface of activated platelets *(37)* through its γ-carboxyglutamic acid domain (K_d ∼320 nM, ∼15 000 sites per platelet), we postulate that a higher-affinity intermediate complex is formed whereby factor XI is initially bound to platelets through sites exposed on the Kringle II domain of prothrombin. Alternatively, a similar initial intermediary complex between factor XI and HK can mediate the initial binding of factor XI to platelets. Both of these interactions are postulated to result in a conformational transition in factor XI resulting in the exposure of a binding site (Asn 235−Arg 266) within the A3 domain of factor XI that mediates a direct, high-affinity interaction with the platelet surface. Platelet-bound factor XI can then be activated efficiently by thrombin.

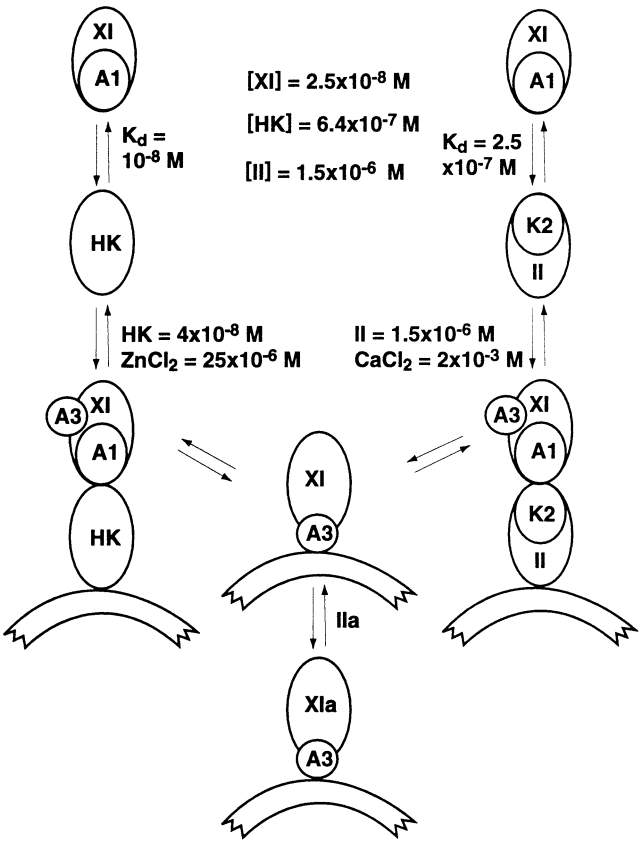

FIGURE 7: Schematic diagram of the role of prothrombin and HK in factor XI binding to activated platelets and thrombin-mediated factor XI activation. See text for explanation. The abbreviations include: XI, factor XI; HK, high molecular weight kininogen; II, prothrombin; A1, Apple 1 domain; A3, Apple 3 domain; K2, Kringle II domain; Iia, thrombin; XIa, factor XIa.

In summary, the data presented in this paper support the conclusion that activated platelets can provide a physiologically relevant surface in vivo for the activation of factor XI by thrombin, thereby explaining the absence of hemostatic deficiency in patients with deficiencies of factor XII, prekallikrein, and HK. Furthermore, the suggestion that this reaction could occur in the complex milieu of human plasma is supported by our observation that prothrombin (at physiological concentration) can abrogate the inhibitory effect of HK (at physiologic concentration) specifically by the capacity of its Kringle II domain to displace HK from its binding site in the A1 domain of factor XI and by the capacity of prothrombin to promote factor XI binding to activated platelets in the presence of Ca^{2+} ions. As previously suggested, the binding of HK or thrombin to the A1 domain of factor XI could regulate the flux of factor XI activation through the contact phase (when HK occupies this site) or through contact-independent feedback activation (when thrombin occupies this site). The presence of prothrombin or PF1.2 at physiological concentrations would favor the latter mechanism, thus supporting the hypothesis *(8, 9)* that the activation of the intrinsic pathway in a revised model of blood coagulation occurs under physiological conditions when thrombin is initially generated via the tissue factor pathway and can then proteolytically activate factor XI by mechanisms independent of the contact phase of coagulation.

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