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Thrombin Activation of Factor XI on Activated Platelets Requires the Interaction of Factor XI and Platelet Glycoprotein Ibα with Thrombin Anion-binding Exosites I and II, Respectively*

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Activation of factor XI (FXI) by thrombin on stimulated platelets plays a physiological role in hemostasis, providing additional thrombin generation required in cases of severe hemostatic challenge. Using a collection of 53 thrombin mutants, we identified 16 mutants with <50% of the wild-type thrombin FXI-activating activity in the presence of dextran sulfate. These mutants mapped to anion-binding exosite (ABE) I, ABE-II, the Na^+-binding site, and the 50-insertion loop. Only the ABE-II mutants showed reduced binding to dextran sulfate-linked agarose. Selected thrombin mutants in ABE-I (R68A, R70A, and R73A), ABE-II (R98A, R24[illegible] and K248A), the 50-insertion loop (W50A), and th[illegible] binding site (E229A and R233A) with <10% [illegible] type activity also showed a markedly red[illegible] activate FXI in the presence of stimul[illegible] ABE-I, 50-insertion loop, and Na^+[illegible] had impaired binding to FX[illegible] glycocalicin, the solubl[illegible] (GPIbα). In contrast, t[illegible] in binding to glyc[illegible] to FXI. Our data su[illegible] thrombin activation[illegible] Thrombin bound to o[illegible] its posterior surface, is[illegible] tion of FXI bound to a r[illegible] ABE-I on its anterior surf[illegible] in the co-localization of th[illegible] sultant efficient activation o[illegible]

Human factor XI (FXI),[1] unique a[illegible]ation proteases in the clotting cascade, circula[illegible]d in zymogen form as a disulfide-linked homodimer (1–3) in a noncovalent complex with high molecular weight kininogen (HMWK) (4). Each FXI monomer is composed of an N-terminal heavy chain and a C-terminal light cha[illegible] containing the serine protease catalytic triad (1, 2). T[illegible]vy chain has four homologous subunits called Ap[illegible] designated A1, A2, A3, and A4, from the N te[illegible]ot found in other coagulation proteases[illegible]kallikrein (3). Additionally, FXI [illegible]with the exception of FXII [illegible]ain "Gla domains," se[illegible]lutamic acids required [illegible]despite the absence [illegible]t with phospholip[illegible]lets in a specific [illegible]istinctive struc[illegible]f activation of [illegible]coagulation [illegible]proteases that can ac[illegible]rombin (8, 9), all of which [illegible]bond and expose the active-site [illegible]mponent of the contact phase of blood [illegible]y to be a physiologically relevant activa[illegible]deficiency is not associated with clinical bleed[illegible] Recent data suggest that thrombin is the physio[illegible]activator of FXI on the activated platelet (12–14). [illegible]rmal hemostasis is initiated by the exposure of tissue factor at sites of vascular injury, followed by the formation of the FVII-tissue factor complex and the subsequent activation of FX. The resultant initial burst of thrombin generation is limited by the rapid and potent inhibition of the FVIIa-tissue factor-FXa complex by the tissue factor pathway inhibitor, but is sufficient to activate platelets, FVIII, FV, and FXI. The subsequent initiation of the consolidation (or intrinsic) pathway of coagulation by thrombin-mediated FXI activation on the activated platelet membrane leads to the sequential activation of FIX, FX, and prothrombin, generating a large secondary burst of thrombin. Thus, FXI activation is required for normal hemostasis only in cases of significant hemostatic challenge, which is consistent with clinical observations that patients with congenital FXI deficiency generally have much milder bleeding than those with FVIII deficiency (hemophilia A) (15, 16). Consistent with these observations is the demonstration that thrombin is more efficient than FXIIa or FXIa in activating FXI *in vitro* (13).

In a current model of FXI activation, the circulating FXI dimer is complexed with either HMWK (17–19) or prothrombin (18, 20), both of which bind to the A1 domain of FXI (21).

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[1] The abbreviations used are: FXI, Factor XI; HMWK, high molecular weight kininogen; GPIb, glycoprotein Ib; ABE, anion-binding exosite; PAR1, protease-activated receptor-1; WT, wild-type; PPACK, D-phenylalanylprolylarginyl chloromethyl ketone; BSA, bovine serum albumin.

Binding of HMWK or prothrombin appears to be required for the exposure of amino acids on the A3 domain of FXI, which binds to the glycoprotein Ib (GPIb)-IX-V complex on the activated platelet surface (12, 22). The FXI homodimer binds to GPIbα in the GPIb-IX-V complex through one of its monomers (12, 14, 23). Thrombin then binds to the A1 domain and activates FXI (20, 24). The binding site on thrombin for FXI has yet to be defined.

The active site of thrombin lies within a deep cleft (25, 26). Access to the active site is restricted by two surface loops, the 50-insertion loop (Leu^{45}–Asn^{57}, thrombin B chain numbering system) and the autolysis loop (Leu^{144}–Gly^{155}), that are situated on the northern and southern rims of the active-site cleft, respectively. Enzyme specificity is further defined by two ligand-binding sites (exosites) that are characterized by a high density of solvent-exposed basic residues. Many thrombin substrates, receptors, and inhibitors gain access to the active site by binding to either of the two exosites. For example, anion-binding exosite (ABE) I is important for binding to fibrinogen (27), fibrin (28), heparin cofactor II (29), PAR1 (30), thrombomodulin (31), and hirudin (32, 33), whereas ABE-II is involved in binding to platelet GPIbα (34), protease nexin I (35), and glycosaminoglycans such as heparin (14).

Utilizing a collection of thrombin mutants generated by alanine scanning site-directed mutagenesis, we previously mapped the interactions of thrombin with some of its key substrates, including protein C, thrombin-activable fibrinolysis inhibitor, and thrombomodulin (36), fibrinogen (37), FV and FVIII (39). In this study, we used this collection bin mutants to identify the key residues on thr for FXI activation. Our data suggest a mod vation of FXI that requires the engage and ABE-II by FXI and the p respectively.

EXP

Materials—Purified h Hematologic Technologies purification of wild-type (W bins from stably transfected been described in detail previo thrombin molecules was determ prolylarginyl chloromethyl keto chromogenic substrate *H*-D-Val-Le mogenix). A comparison of the cata and mutant thrombins toward the chr Arg-*p*-nitroanilide (S-2388, Chromogen (36, 38, 39). Dextran sulfate (average *M* mide-activated Sepharose 4B-agarose we from Sigma. Dimethylmethylene blue was obtained fr a Fine Chemicals (Long Island, NY). Active site-inhibited thrombin was prepared by incubation of a 10-fold excess of PPACK with thrombin for 1 h at 37 °C. This mixture was then dialyzed in Spectrapor tubing (M_r cutoff of 3500; Spectrum Medical Industries, Inc., Los Angeles, CA) overnight in phosphate-buffered saline at 4 °C. The thrombin receptor activation peptide (SFLLRN-amide) was synthesized on an Applied Biosystems 430A synthesizer and purified to >99% homogeneity by reverse-phase high pressure liquid chromatography.

Radiolabeling of Factor XI and PPACK-Thrombin—Purified FXI and PPACK-thrombin were radiolabeled with ^{125}I by a minor modification (20) of the IODO-GEN method to specific activities of 5×10^6 and 2×10^6 cpm/μg, respectively. Radiolabeled FXI retained >98% of its biological activity.

Preparation of Glycocalicin—Glycocalicin was extracted from human platelets and purified as described previously (40).

Factor XI Activation in the Presence of Dextran Sulfate—Mutant thrombins were screened for their ability to activate FXI using a two-stage chromogenic assay. Initially, 1 nM thrombin was incubated with 60 nM FXI for 10 min at 37 °C in 100 μl of assay buffer (50 mM Tris (pH 7.3), 150 mM NaCl, 0.1% bovine serum albumin (BSA), and 1 μg/ml dextran sulfate) to activate FXI. Thrombin activity was inhibited with 25 units/ml hirudin. FXIa activity was measured by combining 20 μl of the reaction mixture with 80 μl of 600 μM S-2366 and observing the change in absorbance at 405 nm on a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA). For 10 mutants demonstrating diminished (<50% compared with WT thrombin) FXI activation, dose-response curves were constructed over a range of thrombin concentrations using the above protocol. Linear initial rates of FXIa generation with respect to time and enzyme (thrombin) concentration were observed in the presence of dextran sulfate as previously reported (8, 9, 13). EC_{50} values were calculated using SigmaPlot software.

Preparation of Washed Platelets—Platelets were prepared as described (6, 14, 23). Platelet-rich plasma obtained from citrated human blood was centrifuged, and the platelets were resuspended in calcium-free Hepes/Tyrode's buffer (126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.38 mM NaH_2PO_4, 5.6 mM dextrose, 6.2 mM sodium Hepes, 8.9 mM Hepes (free acid), and 0.1% BSA) at pH 6.5 and gel-filtered on a column of Sepharose 2B equilibrated in calcium-free Hepes/Tyrode's buffer (pH 7.2). Platelets were counted electronically with a Coulter counter (Coulter Electronics, Hialeah, FL).

Assay of Factor XI Activation in the Presence of Activated Platelets—Activation of FXI (60 nM) by thrombin (and thrombin mutants) was measured by chromogenic assay. Incubations were carried out at 37 °C in 200 μl of 50 mM Tris and 150 mM NaCl (pH 7.3) with 1% BSA and gel-filtered platelets (activated by incubation at 37 °C for 1 min with the thrombin receptor activati de SFLLRN-amide at 25 μM) in the presence of 42 nM HM $_2$, and 25 μM $ZnCl_2$. After dilution to a final volume Tris and 150 mM NaCl (pH 7.3) with 1% BSA the amount of free *p*-nitroanilide was hanges in absorbance at 405 nm end of the reaction to inhibit ctivity after 5-fold dilution led FXI were subtracted surement of only FXIa amount of FXIa generated. ve constructed using ion with respect to erved in the presence of . Dose-response centrations to by the 10 thrombin inished FXI activation

ssay—Dextran sulfate (average ogen bromide-activated Sepharose anufacturer's instructions. 3 g of Washed 0 ml of 10 mg/ml dextran sulfate in coupling ature for 2.5 h. Bound dextran sulfate was quantified by methylene blue assay (35) and determined to be 3.2 n/mg of resin. WT and mutant thrombins were screened for ity to bind to dextran sulfate cross-linked to Sepharose 4B-rose. 500 μl of 10 nM WT or mutant thrombin solution was incubated at room temperature with 50 μl of a 1:2 slurry of dextran sulfate-cross-linked agarose. After 10 min, the mixtures were filtered through spin columns (2000 × *g*), and the resultant flow-through fractions were assayed for thrombin activity using the chromogenic substrate S-2238. 100 μl of flow-through fractions was incubated for 10 min with 100 μl of 0.4 mM S-2238, and the absorbance at 405 nm was measured at that point. Absorbance readings were determined to be linear within the prescribed thrombin concentration ranges. To quantify the amount of bound thrombin, these measurements were subtracted from the $A_{450\ nm}$ of the flow-through fractions from an incubation using non-cross-linked Sepharose 4B. This value was then divided by the $A_{405\ nm}$ of the flow-through fractions from the non-cross-linked Sepharose 4B incubation to obtain a percentage of thrombin bound specifically to dextran sulfate.

Solid-phase Binding of ^{125}I-PPACK-Thrombin to Glycocalicin—We utilized a modified method of De Cristofaro *et al.* (41) to examine the binding of ^{125}I-PPACK-thrombin to plate-bound glycocalicin. Wheat germ lectin (10 μg/ml) was coated on the wells of 96-well polystyrene trays (Immulon high protein capacity binding) and incubated overnight at 4 °C in 50 mM carbonate buffer (pH 9.50). The remaining binding sites in the sample wells were blocked by incubation with 1% BSA in Hepes-buffered saline for 2 h. After aspiration of the BSA solution, purified glycocalicin was added to the wells at a concentration of 20 μg/ml and incubated at 4 °C for 1 h. After aspiration, ^{125}I-PPACK-thrombin was applied to the wells and incubated for 1 h at 37 °C. Each sample and blank well were washed with Hepes-buffered saline seven times for 1 min each, dried, and counted in a Wallac Wizard 1470 γ-counter.

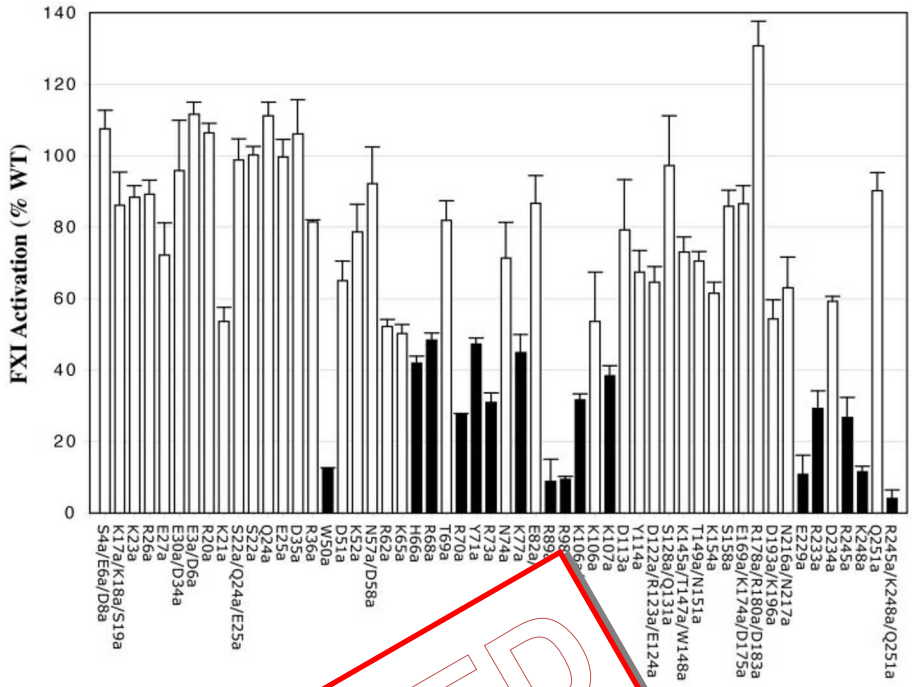

FIG. 1. **Screen of mutant thrombin FXI-activating ability.** FXI activation by thrombin mutants was carried out in the presence of dextran sulfate using a two-stage chromogenic assay as described under "Experimental Procedures." The ability of mutant thrombins to activate FXI in the presence of dextran sulfate is shown as a percentage relative to that of WT thrombin. Results represent the means ± S.D. of at least three separate experiments, each done in duplicate. Mutants averaging <50% relative activating ability are indicated by *black bars*. Thrombin numbering is based on the thrombin B chain.

Solid-phase Binding of ^{125}I-PPACK-Thrombin to Factor XI—We
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(pH 9.50). The remaining binding sites of the
for 2 h with 1% BSA in Hepes-buffered
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displaced 50% of the bound ^{125}
plotting the amount of ^{125}I-thrombi
the amount of competitor ligand ad

RESULT

*Screening for Thrombin Mutants
fate-mediated Factor XI Activation*—A
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sis strategy was used to determine res
on thrombin im-
portant for activating FXI in the presence of dextran sulfate. A collection of 53 thrombin mutants in which solvent-exposed polar and charged residues were mutated to alanine was screened for FXI-activating ability in the presence of dextran sulfate (Fig. 1). A total of 16 mutants mapped to ABE-I, ABE-II, the Na^+-binding site, and the 50-insertion loop demonstrated <50% of the WT thrombin FXI-activating ability. Among these are seven ABE-I mutants, H66A, R68A, R70A, Y71A, R73A, K107A, and the double mutant K106A/K107A, with relative activating abilities ranging from 28 to 48% of that of WT thrombin. Five ABE-II mutants, R89A/R93A/E94A, R98A, R245A, K248A, and R245A/K248A/Q251A, showed greater deficiency in FXI activation, as their relative abilities were all <27%. Interestingly, the effects of the R245A and K248A mutations seem to be additive because the triple mutant R245A/K248A/Q251A displayed an activating ability of 3%, well less than that of either the R245A or K248A mutant (27 and 11%, respectively). Mutation of Gln^{251} does not contribute to the

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entify region(s) of thrombin responsible for
xtran sulfate, thrombin mutants were screened
bility to bind to dextran sulfate-cross-linked agarose
2). Only four mutants bound to dextran sulfate-agarose
with 50% or less efficiency. These four mutants all mapped to ABE-II: R89A/R93A/E94A (45.6 ± 3.6%), R98A (34 ± 1.3%), K248A (50.6 ± 1.1%), and R245A/K248A/Q251A (23.7 ± 2.0%). All of the other mutants bound to dextran sulfate with percentages ranging from 55 to 80%, with WT thrombin binding at 76.7 ± 1.0%. As in the FXI activation screen, the ABE-II triple mutant R245A/K248A/Q251 demonstrated the largest deficiency compared with WT thrombin, and this deficiency was a result of the cumulative effects of the mutations at Arg^{245} and Lys^{248}, but not at Gln^{251}. These data clearly implicate ABE-II as the region responsible for binding to dextran sulfate and suggest that the impairment in FXI-activating ability found among the ABE-II mutants is due solely to inefficient binding to dextran sulfate.

Thrombin Activation of Factor XI in the Presence of Dextran Sulfate—Dose-response curves were generated for 10 of the 16 thrombin mutants found deficient in activating FXI in the initial screen. The selection of these 10 single mutants is representative of the regions identified to be important in FXI activation from the initial activation screen. The EC_{50} values of the mutants corroborate the findings of the initial screen, with all of the mutants demonstrating <10% activating ability over WT thrombin (0.47 ± 0.05 nM). Among these, the ABE-I mu-

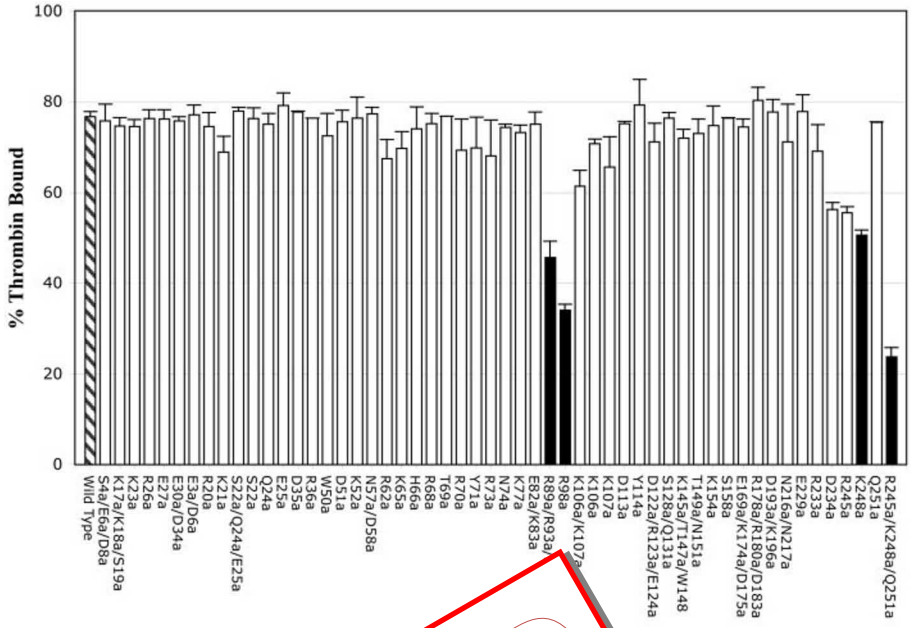


FIG. 2. **Comparison of WT and mutant thrombin abilities to bind to dextran sulfate.** WT and thrombin mutants were incubated with either non-cross-linked or dextran sulfate-cross-linked agarose, and the residual free thrombin activities were determined by S-2238 hydrolysis as described under "Experimental Procedures." Results are expressed as percent thrombin-bound (means ± S.D. of at least three separate experiments, each done in duplicate). Mutants that bound with ≤50% efficiency are indicated by *black bars*, and WT thrombin is indicated by the *hatched bar*.

tants R68A (15.9 ± 1.9 nM) and R73A (15.5 ± 1.1 nM), the … activated with PPACK ABE-II mutant R98A (22.5 ± 3.0 nM), and the 50-insertion l… ding to FXI in a solid-mutant W50A (23.0 ± 4.4 nM) demonstrated at least … ^{125}I-PPACK-thrombin increase compared with WT thrombin.

Factor XI Activation in the Presence of … lets—To identify thrombin residues imp… —The same 10 tion under physiological conditions … ng assay were EC_{50} values for the same 10 d… hase competi-generated using activated … d reduced binding HMWK, 2 mM CaCl… red with WT thrombin, sulfate in the assay … ction (Fig. 5 and Table II). presence of activated … sted showed enhancement of or those obtained from st… binding ability compared with WT sulfate. A notable excep… binding site mutants E229A and R233A tween the EC_{50} values fo… tion loop mutant W50A were also found to be activated platelets (0.1 ± 0.… FXI binding. Taken together, these results indicate fate (0.47 ± 0.05 nM). This d… olvement of ABE-I, the Leu^{45}–Asn^{57} loop, and the Na^+-higher affinity of WT thrombin … ding site in direct interactions with FXI. dextran sulfate.

Binding of Thrombin Mutants to … cin, the extracellular domain of GPIbα, … vered to accelerate thrombin activation of FX… e similar to that observed with activated platelet… . Because GPIbα appears to be the platelet receptor for FXI co-localization with thrombin, the same 10 single thrombin mutants used in the dose-response studies were tested for their ability to competitively inhibit the binding of ^{125}I-PPACK-thrombin to glycocalicin in a solid-phase assay as a measure of their ability to bind to GPIbα. Of these thrombin mutants tested, only the three ABE-II mutants R98A, R245A, and K248A demonstrated a significant reduction in their ability to bind to glycocalicin as demonstrated by their IC_{50} values (Fig. 3 and Table II). All three ABE-II mutants showed at least a 100-fold decrease in binding affinity, whereas the other mutants displayed comparable or enhanced binding to glycocalicin compared with WT thrombin. These data clearly corroborate the findings of the dextran sulfate binding screen and indicate the functional similarity of dextran sulfate and glycocalicin in the activation of FXI.

Binding of Thrombin to Factor XI—To establish an experimental basis for determination of thrombin exosites that me-

DISCUSSION

Thrombin activation of FXI plays an important role in amplifying thrombin generation at sites of vascular injury. In this study, utilizing a collection of thrombin mutants, we have identified the key thrombin residues important in this process (Fig. 6). We used two assay methods for FXI activation by thrombin. The initial screening was performed in the presence of dextran sulfate because it is technically more straightforward (8). The key residues identified were further characterized by dose-response studies, and their importance was confirmed by FXI activation in the presence of activated platelets. Although dextran sulfate is commonly employed in studies of FXI activation by thrombin (8, 9, 20), it should be noted that activated platelets and dextran sulfate may promote thrombin-catalyzed FXI activation by similar but non-identical mechanisms because the binding site for the activated platelet surface has been localized to the A3 domain of FXI (14, 23), whereas binding to dextran sulfate is mediated through the A1 domain (24).

Despite these differences, the key thrombin residues identified to be important in FXI activation in the presence of dextran sulfate were confirmed by the subsequent activated plate-

TABLE I

Thrombin Activation of FXI in the presence of dextran sulfate and activated platelets

10 mutant thrombins identified with impaired FXI-activating ability from the activation screen were selected for dose-response studies. Various concentrations (0.2–50 nM) of WT and mutant thrombins were used in the dextran sulfate or activated platelet (42 nM HMWK, 2 mM $CaCl_2$, and 25 μM, $ZnCl_2$)-supplemented FXI activation assays described under "Experimental Procedures" to generate dose-response curves. EC_{50} values were calculated using SigmaPlot 2000 software. The inverse of mutant EC values standardized to that of WT thrombin was used to generate a comparative measure of FXI-activating ability (% relative to WT). Results represent the means ± S.D. of at least two separate experiments, each done in duplicate.

Thrombin mutation	Location of residue on thrombin	Dextran sulfate		Activated platelets	
		EC_{50}	Relative to WT	EC_{50}	Relative to WT
		nM	%	*nM*	%
WT		0.47 ± 0.05		0.1 ± 0.01	
R68A	ABE-I	15.9 ± 1.9	2.9	9.0 ± 0.8	1.1
R70A	ABE-I	12.2 ± 0.4	3.8	16.5 ± 1.5	0.6
Y71A	ABE-I	8.3 ± 0.7	5.7	11.5 ± 1.05	0.8
R73A	ABE-I	15.5 ± 1.1	3.1	23.5 ± 2.1	0.4
R98A	ABE-II	22.5 ± 3.0	2.1	22.5 ± 2.4	0.44
R245A	ABE-II	5.8 ± 1.4	8.2	7.8 ± 0.6	1.2
K248A	ABE-II	12.7 ± 0.9	3.7	15.1 ± 1.8	0.6
W50A	Leu^{45}–Asn^{57} loop	23.0 ± 4.4	2.0	9.5 ± 0.98	1.0
E229A	Na^+ site	11.7 ± 2.3	4.1	10.5 ± 1.3	0.9
R233A	Na^+ site	8.7 ± 1.3	5.4	18.0 ± 2.1	0.5

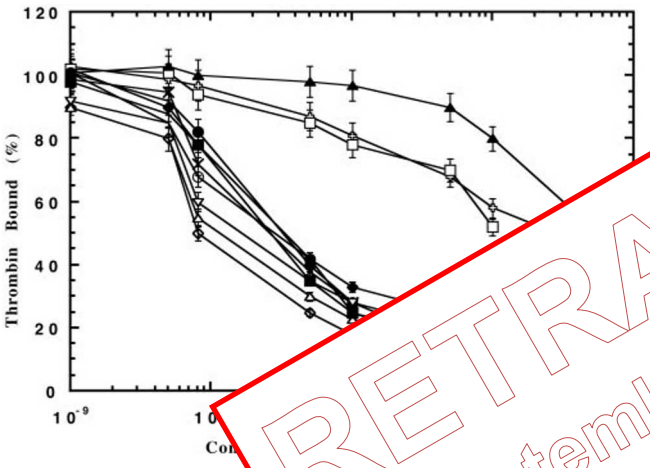


FIG. 3. **Binding of ^{125}I-I… presence of various conc… bins.** ^{125}I-PPACK-thrombin (2… thrombins. When glycocalicin w… plates, the amount of ^{125}I-PPAC… value, and the maximum variation… imental observation was <2% of … binding of ^{125}I-PPACK-thrombin rep… any added WT or mutant thrombin (n… binding as represented by ^{125}I-PPACK-t… coated with BSA (mean = 404 cpm) was subtra… binding at each data point. Results shown represent… ± S.E. of three experiments, each done in duplicate to de… the effects of WT thrombin (○) and thrombin mutants R68A (A…E-I) (■), R70A (ABE-I) (▽), Y71A (ABE-I) (△), R73A (ABE-I) (●), R98A (ABE-II) (▲), R245A (ABE-II) (□), K248A (ABE-II) (✣), W50A (Leu^{45}–Asn^{57} loop) (◆), E229A (Na^+-binding site) (×), and R233A (Na^+-binding site) (◇) on the binding of ^{125}I-PPACK-thrombin to glycocalicin.

let assay system (Table I). All four major functional domains of thrombin were found to be involved in the process, including ABE-I, ABE-II, the 50-insertion loop, and the Na^+-binding site (Fig. 6). The specific roles of the exosites in the FXI interaction were delineated by solid-phase binding experiments using mutants that were found to be defective in activating FXI in the initial activation screen. Four ABE-I single mutants with impaired FXI-activating ability (R68A, R70A, Y71A, and R73A) were chosen for further characterization and found to be deficient in the activated platelet assay system (Table I) and in binding to FXI (Fig. 5 and Table II), but demonstrated normal binding to glycocalicin (Fig. 3 and Table II). These single mutations within ABE… indirectly alter… thrombin… thro… tly disrupt specific contacts or f ABE-I for the interaction of tallographic studies on the solve this issue.

hree ABE-II single mu- ity (R98A, R245A, and nt in binding to FXI major impairment II). These results II in binding to found the fol- g to GPIbα: se residues were ding screen, suggest- FXI activation. Further- esidues tested, substitution of with alanine does not result in tran sulfate or to GPIbα (34). This con- ctrostatic effects in ABE-II binding to GPIbα, d by its salt dependence (42). Whether binding to hosterically modifies the structure of thrombin is still question because conflicting results have been published indicating no effect on PAR1 peptide hydrolysis (42), but inhibitory effects on fibrinopeptide A release and D-Phe-Pro-Arg-*p*-nitroanilide hydrolysis (43) and, more recently, FVIII activation (44).

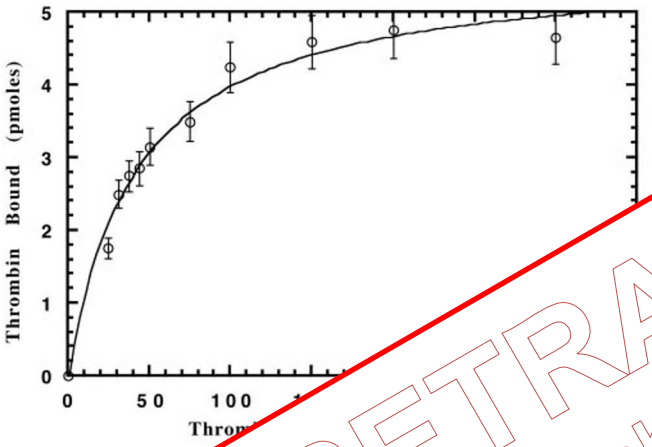
The present data indicate that Trp^{50} in the 50-insertion loop is important in mediating FXI activation in the presence of dextran sulfate or activated platelets (Table I). Substitution of alanine for Trp^{50} had no effect on thrombin binding to glycocalicin (Fig. 3 and Table II), but resulted in severe impairment of thrombin binding to FXI (Fig. 5 and Table II). Trp^{50} plays an important role in defining the apolar S2 subsite and has been shown to make substantial contacts in the thrombin complex with direct thrombin inhibitors such as hirudin (32), hemadin (45), and PPACK (26) as well as with fibrinopeptide A (46). Alanine substitution of Trp^{50} significantly perturbs thrombin activity with all of the substrates tested thus far, disrupting fibrinogen clotting and the activation of protein C, thrombin-activatable fibrinolysis inhibitor, FV, and FVIII (36–39, 47). However, it does not affect the ability of thrombin to bind the DNA thrombin aptamer or fibrin, both of which bind to a defined site in ABE-I (37, 47), suggesting that the insertion loop and the ABE-I site are functionally independent. The significant impairment of FXI activation by the W50A mutant

TABLE II

Effect of thrombin mutants on the binding of ^{125}I-PPACK-thrombin to glycocalicin and to FXI in a solid-phase competition assay

10 mutant thrombins defective in activating FXI were tested for their ability to competitively inhibit the binding of ^{125}I-PPACK-thrombin to either glycocalicin or FXI bound to a solid support, and IC_{50} values were calculated as described under "Experimental Procedures." Mutant IC_{50} values were divided by the WT thrombin IC_{50} values to obtain binding percentages relative to WT thrombin Results represent the means ± S.D. of at least two separate experiments, each done in duplicate.

Thrombin mutation	Location of residue on thrombin	Binding to glycocalicin		Binding to FXI	
		IC_{50}	Relative to WT	IC_{50}	Relative to WT
		nM	%	*nM*	%
WT		22 ± 1.7		100 ± 10	
R68A	ABE-I	13.2 ± 1.4	167	200 ± 15	50
R70A	ABE-I	11.5 ± 0.9	191	1000 ± 50	10
Y71A	ABE-I	9.8 ± 0.6	224	1000 ± 40	10
R73A	ABE-I	24.6 ± 1.2	89	2000 ± 70	5
R98A	ABE-II	4400 ± 38	0.2	50 ± 5	200
R245A	ABE-II	1590 ± 21	0.75	75 ± 4.5	133
K248A	ABE-II	3001 ± 25	0.39	150 ± 18	66.6
W50A	Leu^{45}–Asn^{57} Loop	22.4 ± 1.1	98	4000 ± 38	2.5
E229A	Na^+ site	11.5 ± 0.5	191	2500 ± 180	4
R233A	Na^+ site	8.5 ± 0.6	259	1000 ± 100	10



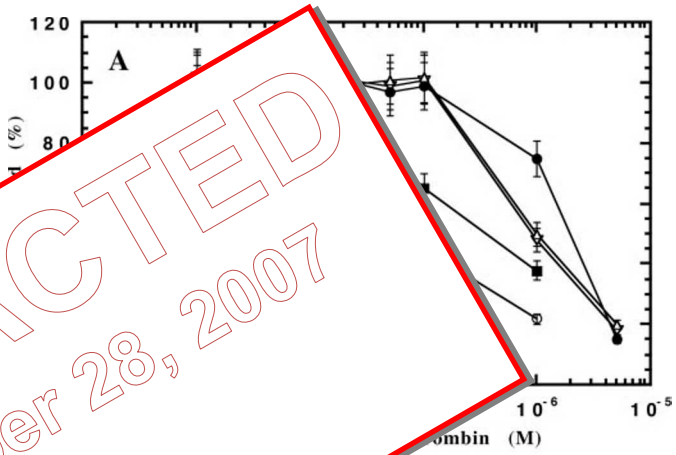


FIG. 4. **Saturable bi**
bound to microtiter plate
various concentrations, a
bound (○) at different input
^{125}I-PPACK-thrombin to unc
and the maximum variation of
observation was <2% of the to
^{125}I-PPACK-thrombin bound to
trol) was subtracted from the tota
the means ± S.E. of three experime

is consistent with either loss of d FXI or disruption of the S2 subsite.

Consistent with all of our previous th n-substrate mapping studies (36–39) is the demonstration in this study of the importance of Glu^{229} and Arg^{233} at the Na^+-binding site in FXI activation (Table I) and in thrombin binding to FXI (Fig. 5 and Table II), whereas in contrast, mutations of the Na^+ site residues (E229A and R233A) led to normal or enhanced binding to glycocalicin (Fig. 3 and Table II). The Na^+-binding site is adjacent to the autolysis loop that forms the southern rim of the active-site cleft (Fig. 6). Na^+-bound thrombin ("fast" form) has a predominantly procoagulant activity, whereas Na^+-free thrombin ("slow" form) has a predominantly anticoagulant property (48–50). Based on empiric screening using this collection of thrombin mutants, we previously identified the thrombin mutants E229A and E229K as essentially anticoagulant forms of the protease with markedly diminished procoagulant properties, but with substantial activity in activating protein C and thrombin-activatable fibrinolysis inhibitor (35, 36, 51, 52). Recently, a new crystal structure of thrombin in which the active site was not occupied was obtained (53). It reveals a

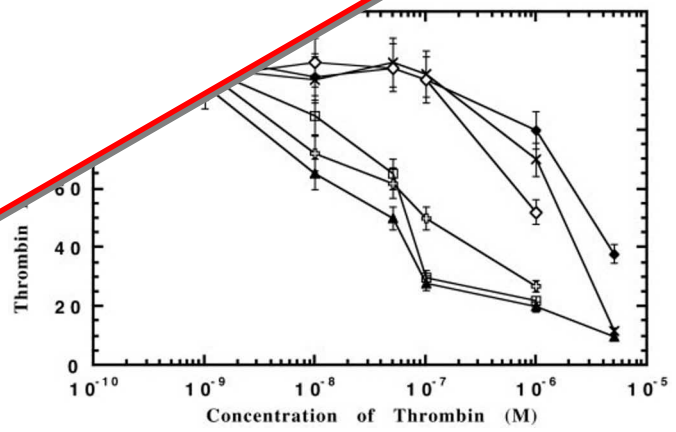


FIG. 5. **Binding of ^{125}I-PPACK-thrombin to FXI in the presence of various concentrations of WT and mutant thrombins.** ^{125}I-PPACK-thrombin (50 nM) was incubated with WT or mutant thrombins. 100% binding of ^{125}I-PPACK-thrombin to FXI represents binding in the absence of any added WT or mutant thrombin (mean = 15,035 cpm). Nonspecific binding as represented by ^{125}I-PPACK-thrombin binding to wells coated with BSA (mean = 301 cpm) was subtracted from the total binding at each data point. ^{125}I-PPACK-thrombin binding to uncoated wells was <0.1% of the total value, and the maximum variation of counts/min bound for each experimental observation was <2% of the total counts/min bound. Results shown in *A* represent the effect of WT thrombin (○) and thrombin mutants R68A (ABE-I) (■), R70A (ABE-I) (▽), Y71A (ABE-I) (△), and R73A (ABE-I) (●). Results shown in *B* represent the effect of R98A (ABE-II) (▲), R245A (ABE-II) (□), K248A (ABE-II) (✣), W50A (Leu^{45}–Asn^{57} loop) (♦), E229A (Na^+-binding site) (×), and R233A (Na^+-binding site) (◇) on the binding of ^{125}I-PPACK-thrombin to glycocalicin. Results shown represent the means ± S.E. of three experiments, each done in duplicate.

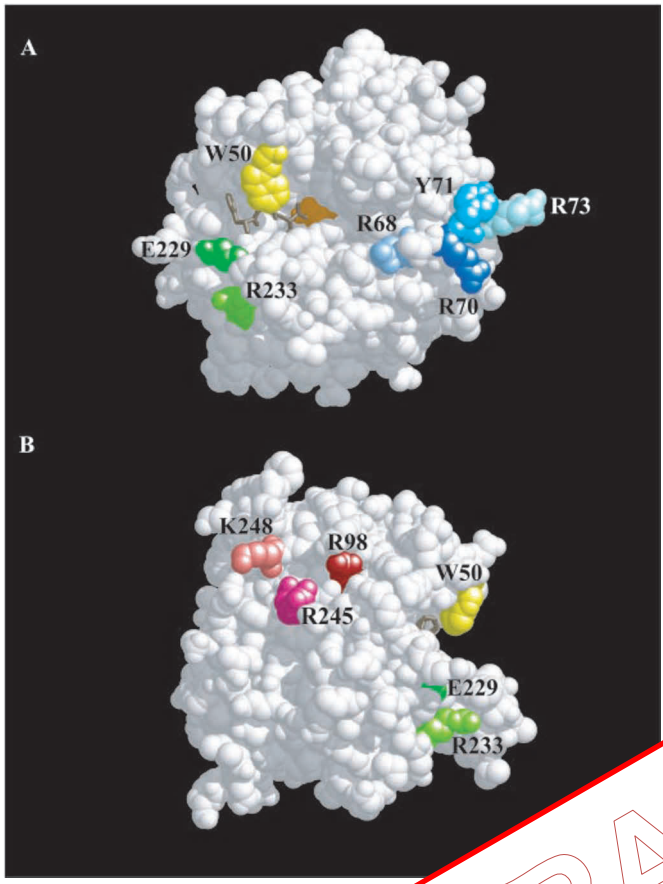


FIG. 6. **Space-filling model of thro… activating FXI.** The Rasmol Version… depict thrombin (EC 3.4.21.5; … space-filling model with solv… be essential for efficient… amino acid code. *A*, the … site cleft running horizo… on the upper rim of the c… The active site is shown wit… a *brown stick model*. Critic… separately in shades of *green*… Trp[50] is colored *yellow*. ABE-I re… are located to the right of the acti… to the left of the cleft. *B*, the same … critical ABE-II residues now visible…

previously unobserved closed confo… …ich satisfies the conditions of the slow … …support-ing the thesis that thrombin can sw… …en these two conformational states. The substantial … FXI activation by the E229A and R233A mutants is consistent with these previous findings. Alanine substitution at the Glu[229] or Arg[233] site may disrupt Na^+ binding and significantly alter the active-site conformation, leading to the closed form of thrombin.

Thus, our current data suggest a quaternary complex model of thrombin activation of FXI on the activated platelet surface. Thrombin binds to the GPIb-IX-V complex via ABE-II on the posterior surface of thrombin and to FXI via ABE-I on its anterior face (Fig. 6). At least one thrombin-binding site on GPIbα has been localized to residues 269–287 (54) and is mediated by electrostatic interactions and perhaps direct contacts. FXI binding to GPIbα through its A3 domain is achieved when one of its homodimers is first complexed with HMWK or prothrombin (14, 23, 24). Once bound to the GPIb-IX-V complex by one of its monomers, FXI can interact with an adjacent thrombin molecule through the A1 domain of its other, free monomer (12). Thus, thrombin bound to one GPIbα molecule, via ABE-II on its posterior surface, is properly oriented for its activation of FXI bound to a neighboring GPIbα molecule, via ABE-I on its anterior surface. In this model, GPIbα plays a major role in co-localizing thrombin and FXI to form the quaternary complex. It would account for the inability of either fibrinogen or HMWK to inhibit this activation in the presence of activated platelets (13).

From our recent thrombin-substrate mapping studies, we found that the binding of thrombin to the GPIb-IX-V complex on the platelet surface is also important in thrombin cleavage of platelet GPV and PAR1.[2] Thus, engagement of thrombin on GPIb-IX-V via ABE-II may facilitate the interaction of thrombin with its physiological substrates on the platelet surface, which may represent the first step in the thrombin-platelet interaction process. The GPIbα-bound thrombin is then in a position to cleave PAR1, GPV, and FXI or to bind another GPIα molecule, all mediated by ABE-I. The specific outcome of this thrombin-substrate interaction will depend on the relative accessibility of these substrates or ligands and other factors that are not yet defined. One notable difference in the activation of FXI by thrombin is that it preferentially occurs only on the activated platelet surface… whereas the cleavage of GPV or PAR1 takes place b… …t activation. Because platelet activation also r… …ntial reduction in the number of GPIb mo… …t surface (55) and redistribution … …em (56), this would imply th… …ted by a new conforma… …y after platelet activa… …ivation leads to redis… …lation of thrombin, …d platelets, which …22). A prediction …ound thrombin …r components …ntly, cleavage …a signaling role in …cleavage of GPV also …I-binding site in the GPIb… …ested.

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