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*

Received for publication, June 29, 2003, and in revised form, September 10, 2003
Published, JBC Papers in Press, September 10, 2003 DOI 10.1074/jbc.M306925200

Thomas H. Yun‡‡, Frank A. Baglia§§, Timothy Myles§, Duraiswamy Navaneetham‡, Jose A. López, Peter N. Walsh**,**, and Lawrence L. K. Leung‡‡‡

From ‡§Division of Hematology, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305, the §Sol Sherry Thrombosis Research Center and the **Departments of Medicine and Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and the [Thrombosis Research Section, Department of Medicine, Baylor College of Medicine, and the Houston Veterans Affairs Medical Center, Houston, Texas 77030

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, R240A, and K248A), the 50-insertion loop (W50A), and Na⁺-binding site (E229A and R233A) with <10% of wild-type activity also showed a markedly reduced ability to activate FXI in the presence of stimulated platelets. ABE-I, 50-insertion loop, and Na⁺-binding site (E229A and R233A) mutants had impaired binding to FXI in the presence of stimulated platelets, while ABE-II mutants were defective in binding to glycocalcin, the soluble form of glycoprotein Ib

** This work was supported by Research Grants HL57530 (to L. L. K. L.), Research Grants HL46213, HL64943, and HL70683 (to P. N. W.), and Research Grant F5065967 (to J. A. L.) from the National Institutes of Health; an American Heart Association scientist development award (to T. M.); the Cheong Har Family Foundation; the Janet Hughes Fund; and the Francis and Arjay Miller Fund (to L. L. K. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† Both authors contributed equally to this work.
‡‡ To whom correspondence should be addressed: Div. of Hematology, Dept. of Medicine, Stanford University School of Medicine, CCSR Bldg., Rm. 1155, 269 Campus Dr., Stanford, CA 94305. Tel.: 650-725-4036; Fax: 650-736-0974; E-mail: lawrence.leung@stanford.edu.

§§ 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 (Leu144–Asn151, thrombin B chain numbering system) and the autolysis loop (Leu114–Gly115), that are situated in 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 GPIb (34), protease nexin I (35), and glycopaminoglycans such as hirapin (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-activatable fibrinolysis inhibitor, and thrombomodulin (36), fibrinogen (37), FXIa (38), and FVIII (39). In this study, we used this collection of thrombin mutants to identify the key residues on thrombin required for FXI activation. Our data suggest a model of thrombin binding to wild-type (WT) and alanine-substituted mutant thrombins toward the chromogenic substrate S-2388 (Chromogenix) has been described in detail (10) of assay buffer (50 mM Tris (pH 8.0) and 150 mM NaCl) with 1% BSA and 0.1% Triton X-100.

EXPERIMENTAL PROCEDURES

Materials—Purified adult human platelets were obtained from laboratories and processing facilities that are certified by the American Red Cross. PPACK-thrombin were radiolabeled with 125I by a minor modification of the method of De Cristofaro et al. (41). We utilized a modified method of De Cristofaro et al. (41) to examine the binding of S-2366-thrombin to plate-bound glycopcalicin. Wheat germ agglutinin (10 µg/ml) was coated on the wells of 96-well polystyrene trays (Immuno high protein capacity binding) and incubated at 4°C for 1 h. After aspiration of the BSA solution, purified glycopcalicin was added to the wells at a concentration of 20 µg/ml. The wells were washed with 125I-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.
Thrombin-Factor XI Interaction

Screening for Thrombin Mutants Defective in Dextran Sulfate—Dextran sulfate binds to Factor XI by acting as a template for the formation of a ternary complex along with the thrombin-FXI interaction (Arg-1107 and Lys-248). Only four mutants bound to dextran sulfate-agarose with 50% or less efficiency. These four mutants all mapped to ABE-II mutants.

RESULTS

Screening for Thrombin Mutants Defective in Dextran Sulfate-mediated Factor XI Activation—A site-directed mutagenesis strategy was used to determine region(s) of thrombin important 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. 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, R245A/R245A/Q251A, K248A, and R245A/K248A/Q251A, showed reduced activity in FXI activation, as their relative activating 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 Gln251 does not contribute to the activating 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.

Solubility Binding of 125I-PPACK-Thrombin to Factor XI—We utilized a modified method of De Cristofaro et al. (41) to examine binding of 125I-PPACK-thrombin to FXI. Wheat germ lectin was coated on the wells of 96-well polystyrene trays (Immulon high protein capacity) and incubated overnight at 4 °C in 50 mM carbonate buffer (pH 9.50). The remaining binding sites of the lectin were blocked with 1% BSA solution in Hepes-buffered saline (pH 7.5). In competition experiments, purified FXI was added to the wells at a concentration of 20 μg/ml and incubated for 1 h at 37 °C. After aspirating the wells, 125I-PPACK-thrombin and the thrombin mutants were applied to the wells and incubated for 1 h at 37 °C with Heps-buffered saline. Wells were washed and counted in a Wallac Wizard 1470 counter. We utilized the Kaleida-graph program (Synergy Software, Reading, PA) to determine the IC50, the concentration of competitor that displaced 50% of the bound 125I-thrombin (IC 50) was determined by plotting the amount of competitor ligand added versus the amount of competitor ligand added. We utilized the Kaleidagraph program (Synergy Software, Reading, PA) to determine the IC50, the concentration of competitor that displaced 50% of the bound 125I-thrombin (IC 50) was determined by plotting the amount of competitor ligand added versus the amount of competitor ligand added.

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 EC50 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.

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 platelet...
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 CaCl₂, and 25 μM, ZnCl₂)–supplemented FXI activation assays described under “Experimental Procedures” to generate dose-response curves. EC₅₀ 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 |
|-------------------|--------------------------------|----------------|--------------------|
|                   |                                | nM             | %                 | nM             | %                 |
| WT                |                                | 0.47 ± 0.05    | 2.9               | 0.1 ± 0.01     | 1.1               |
| R68A              | ABE-I                          | 15.9 ± 1.9     | 3.8               | 16.5 ± 1.5     | 0.6               |
| R70A              | ABE-I                          | 12.2 ± 0.4     | 5.7               | 11.5 ± 1.05    | 0.8               |
| Y71A              | ABE-I                          | 8.3 ± 0.7      | 3.1               | 23.5 ± 2.1     | 0.4               |
| R73A              | ABE-II                         | 15.5 ± 1.1     | 2.1               | 22.0 ± 2.4     | 0.4               |
| R98A              | ABE-II                         | 22.5 ± 3.0     | 8.2               | 7.8 ± 0.6      | 1.2               |
| R245A             | ABE-II                         | 5.8 ± 1.4      | 3.7               | 15.1 ± 1.8     | 0.6               |
| R248A             | ABE-II                         | 12.7 ± 0.9     | 2.0               | 9.5 ± 0.98     | 1.0               |
| W50A              | Leu⁴⁶–Asn⁴⁶ loop                | 23.0 ± 4.4     | 4.1               | 10.5 ± 1.3     | 0.9               |
| E229A             | Na⁺ site                       | 11.7 ± 2.3     | 5.4               | 18.0 ± 2.1     | 0.5               |
| R233A             | Na⁺ site                       | 8.7 ± 1.3      | 5.4               |                   |                   |

Fig. 3. Binding of ¹²⁵I-PPACK-thrombin in the presence of various concentrations of ABE-I and ABE-II mutant thrombins. When glycocalicin was not bound to the wells of microtiter plates, the amount of ¹²⁵I-PPACK-thrombin bound represents binding as represented by ¹²⁵I-PPACK-thrombin binding to wells coated with BSA (mean ± 0.1% of the control). This figure shows that ¹²⁵I-PPACK-thrombin binding to the wells coated with BSA, and any added WT or mutant thrombin (mean ± 0.1% of the control) was subtraced. Results shown represent the mean ± S.E. of three experiments, each done in duplicate to determine the effects of WT thrombin (○) and thrombin mutant ABE-I (△), R70A (△), Y71A (△), R73A (△), R98A (△), R245A (△), R248A (△), W50A (△), E229A (△), Y71A (△), and R233A (△) on the binding of ¹²⁵I-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-I could directly disrupt specific contacts or indirectly alter the local topology of ABE-I for the interaction of thrombin with FXI. Further x-ray crystallographic studies on the thrombin-FXI complex will help to resolve this issue.

Three ABE-II single mutants (R98A, R245A, and R248A) identified in binding to FXI (Table II) were major impairment in FXI activation (Table II). These results further suggest that ABE-I in binding to FXI is more closely related to GPIbα. Additional residues were selected from the initial screening, suggesting that FXI activation. Furthermore, mutating residues tested, substitution of alanine with alanine does not result in reduced binding to dextran sulfate or to GPIbα (34). This could indicate that electrostatic effects in ABE-II binding to GPIbα, as opposed to that achieved by its salt dependence (42). Whether binding to GPIbα allosterically modifies the structure of thrombin is still an open question because conflicting results have been published indicating no effect on PAR1 peptide hydrolysis (42), but inhibitory effects on fibrinopeptide A release and d-Arg-p-nitroanilide hydrolysis (43) and, more recently, FVIII activation (44).

The present data indicate that Trp⁵⁰ 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 Trp⁵⁰ 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). Trp⁵⁰ 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 Trp⁵⁰ 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
Effect of thrombin mutants on the binding of \(^{125}\)I-PPACK-thrombin to glycocalcin 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 glycocalcin 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 glycocalcin | Binding to FXI |
|-------------------|---------------------------------|------------------------|---------------|
|                   |                                 | IC\(_{50}\) \(\mu\text{M}\) | Relative to WT |
|                   |                                 | IC\(_{50}\) \(\mu\text{M}\) | Relative to WT |
| WT                |                                 | 22 ± 1.7                | 100 ± 10      |
| R68A              | ABE-I                           | 13.2 ± 1.4              | 200 ± 15      |
| R70A              | ABE-I                           | 11.5 ± 0.9              | 1000 ± 50     |
| Y71A              | ABE-I                           | 9.8 ± 0.6               | 1000 ± 40     |
| R73A              | ABE-I                           | 24.6 ± 1.2              | 2000 ± 70     |
| R96A              | ABE-II                          | 4400 ± 38               | 50 ± 5        |
| R245A             | ABE-II                          | 1590 ± 21               | 75 ± 4.5      |
| R248A             | ABE-II                          | 3001 ± 25               | 150 ± 18      |
| W50A              | Leu\(^{45}\)–Asn\(^{57}\) Loop | 22.4 ± 1.1              | 4000 ± 38     |
| E229A             | Na\(^{+}\) site                 | 11.5 ± 0.5              | 2500 ± 18     |
| R233A             | Na\(^{+}\) site                 | 8.5 ± 0.6               | 1000 ± 100    |

Values were divided by the WT thrombin IC\(_{50}\) values to obtain binding percentages relative to WT thrombin. Results represent the means ± S.E. of three experiments, each done in duplicate.

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

Consistent with all of our previous thrombin-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 glycocalcin (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
Critical ABE-II residues now visible and colored in shades of red are located to the right of the active-site cleft, whereas ABE-II is located may disrupt Na$^+$ binding and significantly alter the active-site conformation. The substantial loss of FXI activation by alanine substitution at the Glu229 or Arg233 site satisfies the conditions of the slow form of thrombin, supporting the thesis that thrombin can switch between these two conformational states. The substantial loss of 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, which satisfies the conditions of the slow form of thrombin, supporting the thesis that thrombin can switch between these two conformational states. The substantial loss of 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.

REFERENCES

1. Bouma, B. N., and Griffin, J. H. (1977) J. Biol. Chem. 252, 6432–6437
2. Gailani, D., Broze, G. J., Jr. (1991) Science 253, 909–912
3. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) Biochemistry 30, 2417–2424
4. Greengard, J. S., Heeb, M. J., Ersdal, E., Walsh, P. N., and Griffin, J. H. (1986) Biochemistry 25, 3884–3890
5. Suttie, J. W. (1985) Annu. Rev. Biochem. 54, 459–477
6. Greengard, J. S., Heeb, M. J., Ersdal, E., Walsh, P. N., and Griffin, J. H. (1986) Biochemistry 25, 7353–7358
7. Sinha, D., Seaman, F. S., Koshy, A., Knight, L. C., and Walsh, P. N. (1984) J. Clin. Invest. 73, 1550–1556
8. Gailani, D., and Broze, G. J., Jr. (1991) Science 253, 909–912
9. Naito, K., and Fujikawa, K. (1991) J. Biol. Chem. 266, 7353–7358
10. Ragni, M. V., Sinha, D., Seaman, F., Lewis, J. H., Spero, J. A., and Walsh, P. N. (1985) Blood 65, 719–724
11. Rosenthal, R. L., Dreskin, O. H., and Rosenthal, N. (1955) Blood 10, 120–131
12. Baglia, F. A., Badellino, K. O., Li, C. Q., López, J. A., and Walsh, P. N. (2002) J. Biol. Chem. 277, 1662–1668
13. Baglia, F. A., and Walsh, P. N. (2000) J. Biol. Chem. 275, 20514–20519
14. Ho, D. H., Baglia, F. A., and Walsh, P. N. (2000) Biochemistry 39, 316–323
15. Edson, J. R., White, J. G., and Krivit, W. (1967) Thromb. Diath. Haemorrh. 18, 342–348
16. Kitchens, C. S. (1991) Semin. Thromb. Hemost. 17, 55–72
17. Ahmad, S. S., Rawala-Sheikh, R., Cheung, W. F., Jameson, B. A., Stafford, D. W., and Walsh, P. N. (1994) Biochemistry 33, 12048–12055
18. Ho, D. H., Badellino, K., Baglia, F. A., Sun, M. F., Zhao, M. M., Gailani, D., and Walsh, P. N. (2000) J. Biol. Chem. 275, 25139–25145
19. Seaman, F. S., Baglia, F. A., Gury, J. A., Jameson, B. A., and Walsh, P. N. (1994) Biochemistry 33, 12048–12055
20. Baglia, F. A., and Walsh, P. N. (1998) Biochemistry 37, 2271–2281
21. Gailani, D., Ho, D., Sun, M. F., Cheng, Q., and Walsh, P. N. (2001) Blood 97, 3117–3122
22. Baglia, F. A., Shrimpton, C. N., López, J. A., and Walsh, P. N. (2003) J. Biol.

T. Myles, unpublished data.
