Factor VIII C2 Domain Contains the Thrombin-binding Site Responsible for Thrombin-catalyzed Cleavage at Arg^{1689}* 

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Thrombin-catalyzed factor VIII activation is an essential positive feedback mechanism regulating intrinsic blood coagulation. A factor VIII human antibody, A-FF, with C2 epitope, exclusively inhibited factor VIII activation and cleavage at Arg^{1689} by thrombin. The results suggested that A-FF prevented the interaction of thrombin with factor VIII and that the C2 domain was involved in the interaction with thrombin. We performed direct binding assays using anhydro-thrombin, a catalytically inactive derivative of thrombin in which the active-site serine is converted to dehydroalanine. Intact factor VIII, 90-kDa light chain, 72-kDa light chain, and heavy chain fragments bound dose-dependently to anhydro-thrombin, and the Kd values were 48, 150, 106, and 180 nM, respectively. The C2 and A2 domains also dose-dependently bound to anhydro-thrombin, and the Kd values were 440 and 488 nM, respectively. The A1 domain did not bind to anhydro-thrombin. A-FF completely inhibited C2 domain binding to anhydro-thrombin (IC_{50}, 18 nM), whereas it did not inhibit A2 domain binding. Furthermore, C2-specific affinity purified F(ab')2 of A-FF, and the recombinant C2 domain inhibited thrombin cleavage at Arg^{1689}. Our results indicate that the C2 domain contains the thrombin-binding site responsible for the cleavage at Arg^{1689}.

Blood clotting factor VIII (FVIII)^1 is a crucial glycoprotein that accelerates the intrinsic coagulation cascade by acting as a cofactor of factor IXa in the tenase complex (1), and a deficiency of FVIII results in the common hereditary bleeding disorder, hemophilia A. FVIII circulates in plasma as a noncovalent complex with von Willebrand factor (vWF) that stabilizes the synthesis and cofactor activity of FVIII (2–4). Mature FVIII is synthesized as a single chain polypeptide of approximately 300 kDa consisting of 2,332 amino acid residues with a mosaic domain structure arranged in the order of A1-A2-B-A3-C1-C2 (5–7) and secreted into plasma as variable series of heterodimers consisting of the heavy chain (HCh) and the light chain (LCh). The HCh is composed of the A1, A2, and parts of the B domain and exhibits size heterogeneity (90–210 kDa) due to proteolytic cleavage within the B domain. The 90-kDa HCh reflects the absence of the B domain, whereas the 210-kDa species includes the intact B domain. The LCh (80 kDa) corresponds to the A3, C1, and C2 domains. The two chains are noncovalently linked by metal ions between the A1 and A3 domains (6, 7).

FVIII is transformed into an active form by limited proteolysis by two essential serine proteases, thrombin and FXa (8, 9). This procoagulant activity is more pronounced after thrombin activation than after FXa activation (10). Cleavage at Arg^{240} removes the size-heterogeneous B domain from the HCh, producing a 90-kDa HCh fragment, and further cleavage of the 90-kDa HCh fragment (A1-A2) at Arg^{722} between the A1 and A2 domains produces 54- (A1) and 44-kDa (A2) species. Cleavage of the 80-kDa LCh fragment (A3-C1-C2) at Arg^{1689} between the B and A3 domains removes 40 amino-terminal acidic peptides from the A3 domain and produces a 72-kDa fragment. A unique cleavage by FXa at Arg^{1721} produces a 67-kDa LCh fragment. The active form of FVIII is a metal-linked heterotrimer consisting of the A1/A2/A3-C1-C2 domains, lacking the middle B domain (6). Cleavage at Arg^{740} between the A2 and B domains does not contribute to the generation of the FVIII activity. In contrast, cleavages at Arg^{740} and Arg^{1689} are essential for optimal FVIII activity (11–13). Activation of human FVIII at its physiological concentration and at pH 7.4 is followed by a reduction in activity (14). This loss of activity is not caused by further proteolysis but by dissociation of the A2 domain from the active form of FVIII heterotrimer (15).

Thrombin cleavage at Arg^{1689} dissociates FVIII from its complex with vWF (16), and subsequently free FVIII promotes tenase activity on phospholipid (PL) micelles. On the other hand, FXa cleaves only the free form of FVIII, and FXa-catalyzed FVIII activation is completely prevented by the formation of the FVIII-vWF complex (17). Therefore, thrombin cleavage at Arg^{1689} regulates the procoagulant pathway by limiting the association between FVIII and vWF. Thrombin-binding sites within FVIII have not been fully characterized, however.

The C2 domain contains vWF, PL, and FXa-binding sites (18–20) and also includes the common epitopes of human antibodies that develop in hemophilia A patients after replacement therapy and in non-hemophilic individuals (21). In the present study we demonstrated that the C2 domain bound to anhydro-thrombin, a catalytically inactive derivative of thrombin, and also that an anti-FVIII antibody with a C2 epitope that inhibited both the thrombin-catalyzed FVIII activation and

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‡ The abbreviations used are: FVIII, factor VIII; PL, phospholipid; vWF, von Willebrand factor; HCh, heavy chain of FVIII; LCh, light chain of FVIII; FXa, factor Xa; mAb, monoclonal antibody; BU/ml, Bethesda unit/ml; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TSA, human serum albumin; IP, immunoprecipitation.
proteolytic cleavage at Arg1689 blocked thrombin binding to the C2 domain.

EXPERIMENTAL PROCEDURES

Proteins

FVIII was affinity-purified using monoclonal antibody (mAb) NMC-VIII/10 recognizing the FVIII A3 domain (22). The specific activity of purified FVIII was 2,700 units/mg. VWF antigen was not detected in the purified FVIII preparation. LCh and HCh fragments of FVIII, together with the A1, A2, and A3-C1-C2 (thrombin-cleaved 72-kDa LCh) fragments, were prepared from plasma FVIII as described previously (23–25). The recombinant C2 domain was produced and purified according to protocol.2 Purified human α-thrombin (specific activity 3,000 NIH units/mg) was obtained from Sigma.

Anti-FVIII Antibodies

A human antibody, A-FF, was obtained from a multitransfused patient with severe hemophilia A. IgG preparations were fractionated by affinity chromatography on protein A-Sepharose (Amersham Pharmacia Biotech), and F(ab)2 fragments were prepared using immobilized pepsin-Sepharose (Pierce) and protein A-Sepharose. The inhibitor titer was 3,100 Bethesda units/ml (BU/ml) (26). A-FF bound to the LCh fragment of FVIII, and the crucial inhibitory epitope was localized to the amino-terminal acidic region of the A3 domain (22). mAb C5, recognizing the A1 domain, was provided by Dr. C. A. Ci/2 K. Takeshima and K. Fujikawa, submitted for publication.

Neutralization Assay

A-FF F(ab)2 was adjusted to 3 BU/ml and incubated with serial dilutions of FVIII fragments for 2 h at 37 °C. The mixture was further incubated with an equal volume of normal-pooled plasma for 2 h, and the inhibitor titer was measured in the Bethesda assay. All dilutions were prepared using veronal buffer (50 mM sodium acetate, 7 mM sodium barbital, 0.1 M NaCl, pH 7.4), containing 2% human serum albumin (HSA). The percent neutralization was calculated as follows: (1 – inhibitor titer in the presence of FVIII fragment/inhibitor titer in the absence of FVIII fragment) × 100 (%). No FVIII activity was detected in FVIII fragments used in this assay.

Immunoprecipitation (IP) Assay

The binding of each 125I-FVIII fragment to A-FF F(ab)2 was measured quantitatively in an IP assay using protein G-Sepharose (Amersham Pharmacia Biotech) as described previously (21). The IP values (IP unit/ml) were calculated as follows: bound/(total 125I-FVIII fragment – nonspecific) × antibody dilution × 20.

Activation of FVIII by Thrombin

One hundred nM of FVIII diluted in veronal buffer (50 mM sodium acetate, 7 mM sodium barbital, 0.1 M NaCl, pH 7.4) containing 2% HSA was incubated with 15 nM thrombin at 37 °C. At timed intervals, samples (10 µl) were taken from the mixture, and the activation was terminated by 1 mM PMSF, followed by a 1,000-fold dilution with veronal buffer at 4 °C. Each sample was tested for the FVIII activity in a one-stage clotting assay. The presence of thrombin and PMSF in the diluted samples did not affect the FVIII activity in the coagulation assay. For the experiments on the effect of antibody on FVIII activation by thrombin, A-FF F(ab)2 was incubated with FVIII for 2 h at 37 °C prior to the addition of thrombin. F(ab)2 obtained from the plasma of a hemophilia A patient with no inhibitor was used as a control. To determine the neutralizing effect of FVIII fragments on the inhibition of thrombin activation by A-FF, 100 nM of each FVIII fragment was preincubated for 2 h at 37 °C with A-FF F(ab)2 adjusted to 2 BU/ml. The mixtures were then incubated with intact FVIII for 2 h, followed by the addition of thrombin.

Analyses of FVIII Cleavage by Thrombin

SDS-PAGE—Twenty nM 125I-FVIII diluted in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) was incubated with 15 nM thrombin. At timed intervals, samples (20 µl) were taken, and activation was terminated by adding 0.4% SDS and boiling for 5 min. Each sample was analyzed on 7.5% SDS-PAGE, followed by autoradiography of dried gels. For the experiments on the effects of A-FF on thrombin cleavage of FVIII, 0.5 µM of A-FF F(ab)2 was incubated with 125I-FVIII for 2 h at 37 °C prior to the addition of thrombin.

Enzyme-linked Immunosorbent Assay (ELISA) for Evaluation of Thrombin Cleavage at Arg1689—Microrod plates (NUNC, Denmark) were coated overnight at 4 °C with 0.2 µM mAb C5 in 0.1 M sodium bicarbonate, pH 9.6. After blocking with phosphate-buffered saline containing 4% HSA for 2 h at 37 °C, 20 nM FVIII was added to each well and incubated for 2 h, followed by the addition of 15 nM thrombin. At timed intervals, supernatants were removed, and 1 mM PMSF was added to the well at 4 °C to quench the thrombin reaction with the captured FVIII. Bound FVIII was determined by peroxidase-conjugated mAb NMC-VIII/10 recognizing the acidic region of LCh, followed by the addition of o-phenylenediamine dissolved in 25 mM citric acid, 50 mM NaH2PO4, and 0.03% H2O2.H2SO4 (2 M) was added as a quenching agent, and the absorbance was read at 492 nm (Lab System Multiskan Multisoft, Helsinki, Finland). For the experiments on the effects of A-FF on thrombin cleavage at Arg1689 of LCh, serial dilutions of A-FF F(ab)2 or C2-specific F(ab)2 were incubated with FVIII for 2 h at 37 °C prior to adding to the mAb C5-coated wells. The presence of PMSF did not interfere with this assay, and two mAbs did not inhibit thrombin cleavage of FVIII and did not compete with A-FF for FVIII binding (data not shown). The rate of LCh cleavage was calculated as follows: (1 – bound – nonspecific A492/bound at time zero – nonspecific A492) × 100 (%). Absorbance in the absence of FVIII was regarded as nonspecific.

In competitive inhibition experiments using FVIII fragments (72-kDa LCh, HCh or recombinant C2 domain), 0.2 µM of NMC-VIII/10 was coated onto microrod wells as described above. In this assay, 100 nM of each FVIII fragment was added together with thrombin, and peroxidase-conjugated NMC-VIII/5 was used for detection of bound FVIII.

Binding Assay by ELISA Using Immobilized Anhydro-thrombin

Five µg of anhydro-thrombin in TBS, pH 7.4, was immobilized onto each microrod well. After blocking with 4% HSA, serial dilutions of FVIII fragments were added and incubated for 2 h at 37 °C. Bound FVIII was detected by peroxidase-conjugated mAb NMC-VIII/5, JR5, or C5 which did not affect thrombin action. In competitive experiments...
TABLE I

Properties of human antibody A-FF

| FVIII fragments | Neutralization assay | Immunoprecipitation assay |
|-----------------|----------------------|--------------------------|
|                 | %                    | IP units/ml               |
| A1              | <5                   | 0                        |
| A2              | <5                   | 5                        |
| 80-kDa LCh      | >95                  | 1580                     |
| 72-kDa LCh      | >95                  | 1570                     |
| C2              | 59                   | 1120                     |

using FVIII fragments, 50 nm 125I-FVIII and serial dilutions of FVIII fragments were mixed prior to the addition to immobilized anhydrotoglobin. Bound 125I-FVIII was measured in a γ-counter. The percent inhibition was calculated as follows: (1 − (count − nonspecific count)/(maximum − nonspecific)) × 100 (%). The count in the absence of FVIII fragments or 125I-FVIII was regarded as maximum or nonspecific, respectively.

Kinetics Measurement Using Real-time Biomolecular Interaction Analysis

The kinetics of FVIII and thrombin interaction was determined by surface plasmon resonance using a BLACore 2000 instrument (Biacore AB, Uppsala, Sweden). Anhydrotoglobin was covalently coupled to an activated carboxymethyl dextran-coated sensor chip surface at a coupling density of 7 ng/mm². Binding (association) of the ligand was monitored in TBS, pH 7.4, 0.005% Tween 20 at a flow rate of 10 μl/min for 4 min. Dissociation was monitored over a 5–10-min range after return to a buffer flow. After each analysis, regeneration of the chip surface was achieved by 0.1 M glycine, pH 2.0, for 2 min. The association rate constants (kₐ) and dissociation rate constants (kₐ) were calculated by nonlinear regression analysis as described previously (33) using the evaluation software provided by Biacore AB. Equilibrium dissociation constants (Kₑ) were calculated as kₐ/kₐ.

Effect of A-FF on 125I-C2 or 125I-A2 Domain Binding to Anhydrotoglobin

Serial dilutions of A-FF F(ab)² were incubated with 100 nm 125I-C2 or 125I-A2 domain for 2 h at 37 °C and then added to immobilized anhydrotoglobin (5 μg). Bound 125I-C2 or 125I-A2 domain was measured in a γ-counter. The percent inhibition was calculated as follows: (1 − (count − nonspecific count)/(maximum − nonspecific)) × 100 (%). The counts in the absence of A-FF or 125I-C2 and 125I-A2 domain were regarded as maximum or nonspecific, respectively.

RESULTS

The Determination of the Epitope of A-FF—The results of neutralization assays and IP assays for epitope localization of A-FF are summarized in Table I. The neutralization assays, the 80-kDa LCh and 72-kDa LCh fragments completely neutralized (>95%) the anti-FVIII activity of A-FF. The recombinant C2 domain neutralized anti-FVIII activity by 59%. Neither the A1 nor the A2 domain neutralized antibody activity (<5%). In the IP assays, the IP value of the 80-kDa LCh fragment was similar to that of the 72-kDa LCh. The IP value of the C2 domain was approximately 70% that of 80-kDa LCh, whereas no or little reactivity was detected with the A1 or A2 domain. The results indicated that the crucial epitope of A-FF was located within the C2 domain.

Inhibitory Effects of A-FF on FVIII Activation by Thrombin—In control experiments in the presence of F(ab)² from the plasma of a hemophilia A patient without antibody, there was an initial 7–8-fold increase in FVIII clotting activity after 3 min of incubation with thrombin (Fig. 1A). This was followed by a reduction in activity, and the base-line level was reached within 30 min. In the presence of A-FF F(ab)², FVIII activation was inhibited in a dose-dependent manner. At a final concentration of 2 BU/ml, the peak FVIII activity was approximately 10% that seen with the control F(ab)², and base-line levels were reached within 10 min. This inhibitory effect of A-FF on thrombin-induced FVIII activation was further investigated in competitive assays using FVIII fragments (Fig. 1B). The 80-kDa LCh and 72-kDa LCh fragments almost completely neutralized the inhibitory effect of A-FF. The neutralizing ability of the C2 domain was approximately half that of the 80-kDa LCh or 72-kDa LCh, whereas no neutralizing effect was observed using the A2 domain.

Inhibitory Effect of A-FF on FVIII Cleavage by Thrombin—To examine the effect of A-FF on FVIII cleavage by thrombin, 125I-labeled FVIII was incubated with thrombin in the presence of A-FF F(ab)², and proteolytic cleavage patterns were analyzed by SDS-PAGE. In the presence of control F(ab)², the expected conversion of the 90–210-kDa fragments of the HCh into 54- and 44-kDa fragments was observed together with breakdown of the 80-kDa LCh into the 72-kDa fragment (Fig. 2A). In contrast, A-FF completely blocked the cleavage of the 80-kDa LCh (total absence of the 72-kDa LCh) but did not affect the cleavage of the HCh (Fig. 2B). These results indicated that A-FF blocked exclusively thrombin-catalyzed proteolytic cleavage at Arg1689 of LCh.

Quantitation of Inhibitory Effect of A-FF on Thrombin-catalyzed Proteolysis at Arg1689 of LCh—We evaluated more quan-
titatively the inhibitory effect of A-FF on thrombin cleavage at Arg^{1689} of LCh using an ELISA. In this system, we utilized mAbs C5 and NMC-VIII/10 recognizing the A1 and A3 domains, respectively. The mAb NMC-VIII/10 binds to the acidic region of the A3 domain (epitope, residues 1675–1684) but does not bind to the 72-kDa LCh fragment (22). The A1/A2/A3-C1-C2 domain conformation is maintained by metal linkage (6), and thus, the thrombin-catalyzed cleavage at Arg^{1689} can be evaluated by measuring reduction of the reactivity of NMC-VIII/10 with FVIII. Total cleavage (>95%) was observed in the presence of control F(ab){sub}2 (A) or A-FF F(ab){sub}2 (B) (0.5 μM). At timed intervals, samples were taken, and the reaction was stopped by adding 0.4% SDS and boiling. The arrow (*) points to an uncharacterized nonspecific band not visible in any of the preparations using unlabeled FVIII.

Competitive Inhibition of Thrombin Proteolysis of LCh by FVIII Fragments—Since an anti-C2 antibody A-FF inhibited thrombin-catalyzed proteolysis of LCh at Arg^{1689}, we tested the hypothesis that the C2 domain is an essential domain for the interaction between FVIII and thrombin. FVIII fragments (72-kDa LCh, HCh or C2 domain) were examined for their ability to compete for the thrombin-catalyzed proteolysis at Arg^{1689} of LCh in an ELISA described above (Fig. 4). The 72-kDa LCh fragment competitively inhibited the cleavage of LCh by >95%. Similarly the recombinant C2 domain inhibited the cleavage by 69%. However, minimal competitive inhibition (<5%) was observed by the HCh fragment. These findings implicated a direct role of the C2 domain in the thrombin-catalyzed proteolysis of FVIII LCh.

Binding of FVIII Fragments to Immobilized Anhydro-thrombin—In order to examine the interaction between FVIII and thrombin, we performed direct binding assays using the active-site modified thrombin (anhydro-thrombin). Binding of FVIII fragments was detected by mAb NMC-VIII/5, JR8, or C5 which did not affect thrombin reactivity. Control experiments using mAb NMC-VIII/5 or JR8 demonstrated that intact FVIII bound to anhydro-thrombin in a dose-dependent manner (Fig. 5). Furthermore, the 80-kDa LCh and 72-kDa LCh fragments, the recombinant C2 domain, the HCh fragment, and the A2 domain also showed dose-dependent binding patterns. In con-
Figure 4. Competitive inhibition of LCh cleavage by thrombin with FVIII fragments. Each FVIII fragment (100 nM) was mixed with thrombin (15 nM), and then the mixture was added to FVIII (20 nM) bound to immobilized NMC-VIII/10. Thrombin reaction was quenched by 1 mM PMSF at the indicated times. Cleavage of FVIII LCh was detected by peroxidase-conjugated mAb NMC-VIII/5. The symbols used are as follows: 72-kDa LCh, C; 80-kDa LCh, E; HCh, □; no fragment, ■.

Figure 5. Binding of FVIII fragments to immobilized anhydro-thrombin. Serial dilutions of FVIII (50 nM) were added to immobilized anhydro-thrombin (5 μg). Bound FVIII fragments were detected by peroxidase-conjugated mAb NMC-VIII/5 (A) JR8 or C5 (B). The symbols used are as follows; A, intact FVIII; C, 80-kDa LCh; ■, 72-kDa LCh; □, C2 domain; ■, and B, intact FVIII; C, HCh; ■, A2 domain; □; A1 domain, ■.

Contrast, the A1 domain did not bind to anhydro-thrombin (Fig. 5, A and B).

Competitive Experiments for FVIII Binding to Anhydro-thrombin—Competitive effects of FVIII fragments on the binding of 125I-FVIII to anhydro-thrombin are illustrated in Fig. 6. A mixture of the 80-kDa LCh and HCh fragment inhibited completely (>95%) 125I-FVIII binding to anhydro-thrombin. The 80-kDa LCh, 72-kDa LCh, and HCh fragments inhibited 125I-FVIII binding to anhydro-thrombin by 56, 63, and 59%, respectively. The C2 and A2 domains inhibited the binding by 34 and 38%, respectively. The 54-kDa A1 domain, however, showed no inhibition even at a concentration of 1 μM.

Kinetic Parameters for Interaction between FVIII Fragments and Anhydro-thrombin—Kinetic measurements (kₐ, k₈, and Kₐ) for direct binding of FVIII fragments to immobilized anhydro-thrombin were performed using surface plasmon resonance, and the results are illustrated in Table II. The Kₐ value for intact FVIII was 48 nM, and those for the 80-kDa LCh and HCh fragments were 150 and 180 nM, respectively, which were approximately 3-fold higher than that of FVIII. A mixture of the 80-kDa LCh and HCh gave the Kₐ value (52 nM) similar to that of FVIII. The Kₐ values for the 72-kDa LCh, C2, and A2 domains were 106, 440, and 480 nM, respectively. The A1 domain, however, did not bind to anhydro-thrombin confirming the results of the ELISA. Representative curves for FVIII and C2 domain binding to immobilized anhydro-thrombin are shown in Fig. 7, A and B.

Inhibitory Effects of A-FF on 125I-C2 or 125I-A2 Domain Binding to Anhydro-thrombin—The inhibitory effects of A-FF

Table II

| Soluble ligand | kₐ (μM⁻¹ s⁻¹) | k₈ (s⁻¹) | Kₐ (nM) |
|---------------|--------------|-----------|--------|
| FVIII         | 2.5 × 10⁻⁴   | 1.2 × 10⁻³ | 48     |
| HCh + 80-kDa LCh | 2.4 × 10⁻⁴  | 1.2 × 10⁻³ | 52     |
| HCh           | 1.1 × 10⁻⁴   | 2.0 × 10⁻³ | 180    |
| 80-kDa LCh    | 1.2 × 10⁻⁴   | 1.8 × 10⁻³ | 150    |
| 72-kDa LCh    | 1.3 × 10⁻⁴   | 1.4 × 10⁻³ | 106    |
| C2            | 7.0 × 10⁻³   | 3.1 × 10⁻³ | 440    |
| A2            | 6.2 × 10⁻³   | 3.0 × 10⁻³ | 488    |
| A1            | ND*          | ND        |        |

*ND, not detected.

Figure 6. Competitive binding of FVIII fragments and 125I-FVIII to anhydro-thrombin. Serial dilutions of FVIII (50 nM) were mixed prior to addition to immobilized anhydro-thrombin (5 μg). Bound 125I-FVIII was measured in a γ-counter. The symbols used are as follows: 80-kDa LCh, C; 72-kDa LCh, ■; 80-kDa LCh plus HCh, □; C2 domain, □; A2 domain, ●; A1 domain, ■.

Figure 7. Determination of the kinetic parameters for the interaction between FVIII fragments and anhydro-thrombin. Anhydro-thrombin was immobilized on a biosensor chip at 7 ng/mm². The kinetics of intact FVIII (A) and C2 domain (B) association with anhydro-thrombin were monitored at a flow rate of 10 μl/min for 4 min, and those of dissociation were monitored over a 5–10-min range after returning to a buffer flow. The kinetics were studied at ligand concentrations of 0.1, 0.2, and 0.4 μM (A) and 0.25, 0.5, and 1 μM (B) corresponding to curves 1–3.

F(αβ)₉ on 125I-C2 or 125I-A2 domain binding to immobilized anhydro-thrombin are illustrated in Fig. 8. A-FF did not inhibit binding of 125I-A2 to anhydro-thrombin. In contrast, A-FF inhibited dose-dependently the binding of 125I-C2 domain to anhydro-thrombin. Complete inhibition was observed at a concentration of 500 nM, and the IC₅₀ value was 18 nM.

DISCUSSION

A-FF, a human antibody obtained from a multitransfused patient with severe hemophilia A, was a highly potent FVIII
inhibitor. It blocked both thrombin-catalyzed FVIII activation and cleavage. Therefore, we hypothesized that the antibody might recognize crucial regions for the interaction between FVIII and thrombin. Epitope analyses by neutralization assays and IP assays revealed that the major epitope is located within the C2 domain of FVIII, strongly suggesting that the region is associated with thrombin-catalyzed activation of FVIII. The inhibitory effects of A-FF on FVIII and thrombin interaction may be mediated both in direct and indirect manners. The antibody may inhibit thrombin binding to FVIII directly or it may interfere with the thrombin cleavage site of FVIII directly or indirectly by a steric hindrance. Alternatively, it is possible that the amino-terminal acidic region of the A3 domain of FVIII may be closely associated with the C2 domain in the three-dimensional structure. vWF-binding sites are known to be located in both the C2 and A3 domains, and FVIII and vWF association is enhanced by the presence of both regions (34). The present anti-C2 antibody, therefore, might have inhibited thrombin cleavage in the amino-terminal A3 region. In addition, the presence of an epitope in the A3-C1 region could not be excluded by our epitope localization experiments, and such an antibody possibly contributes to inhibit thrombin cleavage. Quantitative kinetic analyses were undertaken, therefore, to closely examine these interactions.

The thrombin-binding site on FVIII has not been identified with certainty, and the relationship between the thrombin-binding site and its catalytic target site for action remains unknown. Experiments using mutant FVIII produced by site-directed mutagenesis together with studies of naturally occurring dysfunctional FVIII molecules from plasma of hemophiliacs have revealed that the mutation of Arg1689 does not affect thrombin cleavage at Arg1689 (11, 12, 35). Similarly, mutation of Arg1689 does not affect a cleavage at Arg1772. These findings strongly suggest, therefore, that proteolytes at Arg1689 and Arg1772 occur independently. In our experiments, A-FF blocked only the cleavage at Arg1689, but not at Arg1772, and C2-specific Fab’ also inhibited the cleavage at Arg1689. Furthermore, LCh and C2 domain competitively inhibited the cleavage at Arg1689 but HCh fragment did not compete, supporting the concept that thrombin-catalyzed reactions are independent at both sites.

Conventional FVIII binding assays using active thrombin are extremely difficult to interpret because activation of FVIII by thrombin-catalyzed cleavage might deplete physiological binding responses. The use of inactive thrombin prepared by modification with several synthetic serine protease inhibitors, such as diisopropyl fluorophosphate, has been considered to minimize enzyme-catalyzed degradation, but conformational change and steric hindrance due to incorporation of diisopropyl phosphate group into the active site of thrombin might disrupt the interaction with FVIII. In the current studies we established a direct binding assay using anhydro-thrombin, a catalytically inactivated thrombin in which the serine residue in the active site was converted to dehydroalanine. The Kd value of FVIII binding to anhydro-thrombin was 48 nm, and the binding pattern was dose-dependent. Thus, although binding to anhydro-thrombin might not be directly equivalent to the physiological interaction, the modified enzyme offered the advantages that the binding experiments could be performed in the absence of proteolytic activity, and steric hindrance due to modification of the active site and conformational changes was minimal.

Both the 80-kDa LCh and HCh fragments bound to anhydro-thrombin in a dose-dependent manner with similar kinetic parameters. The equilibrium dissociation constants of both chains were larger than that of intact FVIII, but the Kd value of a mixture of both fragments was similar to that of the intact FVIII. These findings are consistent with the proposal that thrombin-binding sites on the LCh and HCh are independent of each other although the maximum thrombin binding requires the presence of both chains. The 72-kDa LCh fragment bound to anhydro-thrombin with a higher affinity than that of the 80-kDa LCh, indicating that the thrombin-binding site on the LCh is not located within the amino-terminal acidic region of the A3 domain.

We further demonstrated that the recombinant C2 domain as well as the A2 domain bound to anhydro-thrombin, confirming the presence of binding sites in both domains. The C2 domain is known to contain both vWF- and PL-binding sites (18, 19), although FVIII activation by thrombin is not believed to be influenced by the presence of vWF or PL (15, 36). FVIII binding to anhydro-thrombin in the current investigations was also not affected by the presence of vWF or PL (data not shown), suggesting that the thrombin-binding site in the C2 domain is separated from the vWF- and PL-binding sites. In our previous work (20) we identified the FXa-binding site within the C2 domain, and we demonstrated that binding of FXa was completely inhibited by the presence of the vWF. This is markedly in contrast to the binding profile of thrombin demonstrated in the current study. Therefore, it should be pointed out that the thrombin-binding site does not overlap with the FXa-binding site, although both enzymes cleave the same sites of FVIII.

In general, enzyme-binding sites are located within or close to their respective cleavage sites. Exceptions are often documented, however, and in particular the FVIII-binding site for activated protein C is located in the carboxyl terminus of the A3 domain, whereas the cleavage site lies within the A1 domain (37). Both regions may be conformationally close in the three-dimensional structure. It is not surprising, therefore, that the thrombin-binding site locates in the C2 domain which is remote in a linear sequence from the cleavage site in the acidic region of A3 domain if both regions are in close proximity in the three-dimensional structure of native FVIII. The Kd value for the binding of the C2 domain to anhydro-thrombin was larger than those of the 80- and 72-kDa LCh fragments and similar to that of the A2 domain. Several reasons for this lower affinity of the C2 domain can be considered. One explanation may be that at least the size of 72-kDa LCh is necessary for the intact conformation of the C2 domain. Another possibility is that an alternative thrombin-binding site is present within the A3 or C1 domain. Although these possibilities cannot be excluded,
our present results indicate that both the C2 and A2 domains contain major thrombin-binding sites. The antibody with a C2 epitope inhibited thrombin-catalyzed cleavage only at Arg1689 and inhibited only the binding of the C2 domain to anhydrothrombin. Furthermore the C2 domain competed for thrombin-catalyzed cleavage at Arg1689 but the HCh did not. We conclude, therefore, that the C2 domain contains the thrombin-binding site that is responsible for thrombin-catalyzed cleavage at Arg1689. Further studies are required to characterize fully the mechanism of thrombin binding to FVIII and clarify the reactions leading to thrombin-catalyzed FVIII activation.

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