Role of Factor VIII C2 Domain in Factor VIII Binding to Factor Xa*

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Factor VIII (FVIII) is activated by proteolytic cleavages with thrombin and factor Xa (FXa) in the intrinsic blood coagulation pathway. The anti-C2 monoclonal antibody ESH8, which recognizes residues 2248–2285 and does not inhibit FVIII binding to von Willebrand factor or phospholipid, inhibited FVIII activation by FXa in a clotting assay. Furthermore, analysis by SDS-polyacrylamide gel electrophoresis showed that ESH8 inhibited FXa cleavage in the presence or absence of phospholipid. The light chain (LCh) fragments (both 80 and 72 kDa) and the recombinant C2 domain dose-dependently bound to immobilized anhydro-FXa, a catalytically inactive derivative of FXa in which dehydroalanine replaces the active-site serine. The affinity ($K_d$) values for the 80- and 72-kDa LCh fragments and the C2 domain were 55, 51, and 560 nM, respectively. The heavy chain of FVIII did not bind to anhydro-FXa. Similarly, competitive assays using overlapping synthetic peptides corresponding to ESH8 epitopes (residues 2248–2285) demonstrated that a peptide designated EP-2 (residues 2253–2270; TSMYVKEFLISSQDGHQ) inhibited the binding of the C2 domain or the 72-kDa LCh to anhydro-FXa by more than 95 and 84%, respectively. Our results provide the first evidence for a direct role of the C2 domain in the association between FVIII and FXa.

Factor VIII (FVIII)1 is a glycoprotein cofactor that accelerates the generation of factor Xa (FXa) by factor IXa in the presence of Ca$^{2+}$ and negatively charged phospholipid (PL) expressed on a membrane surface (1). Quantitative and qualitative deficiencies of FVIII result in the congenital bleeding disorder, hemophilia A. FVIII is noncovalently bound to von Willebrand factor (vWF) in plasma. vWF regulates the synthesis, the cofactor activity, and the transport of FVIII to the site of vascular injury (2–4). Mature FVIII is synthesized as a single chain polypeptide consisting of 2332 amino acid residues (5, 6). Based on internal homologies of the amino acid sequence, FVIII has three types of domains arranged in the order of A1-A2-B-A3-C1-C2 (7). FVIII circulates in the plasma as a heterodimer of a heavy chain (HCh) consisting of the A1, A2, and heterogeneous fragments of partially proteolyzed B domains, together with a light chain (LCh) consisting of A3, C1, and C2 domains (6, 7).

Several findings have indicated that the structure and function of the C2 domain is important for the expression and regulation of FVIII. The C2 domain contains a PL binding site (6, 7). Cleavage at Arg$^{72}$ and Arg$^{740}$ of the 90-kDa HCh fragment containing the A1 and A2 domains produces 54-kDa (A1) and 44-kDa (A2) species. Cleavage of the 80-kDa LCh fragment (A3-C1-C2) at Arg$^{1609}$ removes 40 amino-terminal acidic peptides from the A3 domain (16) and produces a 72-kDa fragment. Cleavage by FXa at Arg$^{1721}$ produces a 67-kDa LCh fragment (17). Proteolysis at Arg$^{72}$ and Arg$^{1609}$ is essential for generating FVIIIa cofactor activity (18–20). FXa-dependent FVIII activation is different from thrombin-dependent FVIII activation in several ways (21, 22). The procoagulant activity of FVIIIa produced by FXa is 4-fold lower and is more stable than that generated by thrombin (22). Furthermore, the presence of vWF modulates the activation of FVIII by FXa but not by thrombin (23). Recently, Lapan and Fay (24) localized a factor X (FX) binding site within the A1 domain. The role of FXa-dependent FVIII activation in vivo is still uncertain, however.

In the present study, we demonstrated that an anti-C2 monoclonal antibody, containing an epitope within residues Val$^{2248}$–Gly$^{2260}$, inhibited FXa cleavage of FVIII in the absence of PL. Furthermore, the C2 domain competed with FVIII for FXa cleavage of the LCh and bound directly to immobilized anhydro-FXa, a catalytically inactive derivative of FXa in which dehydroalanine replaces the active-site serine, indicating that the C2 domain contains a FXa binding site.

EXPERIMENTAL PROCEDURES

Proteins

FVIII was affinity-purified using monoclonal antibody NMC-VIII/10, recognizing the FVIII A3 domain. Elution from the monoclonal antibody column was performed with 1 M KI and 40 mM ethylene glycol as described previously (25). The specific activity of the purified FVIII was 2700 units/mg. Enzyme-linked immunosorbent assay (ELISA) demonstrated that the purified FVIII was free of vWF antigen (26). LCh and HCh fragments of FVIII, together with A1, A2, and thrombin-cleaved 72-kDa LCh fragments, were prepared from plasma FVIII as described previ-

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‡ The abbreviations used are: FVIII, factor VIII; FXa, factor Xa; PL, phospholipid; vWF, von Willebrand factor; HCh, heavy chain of FVIII; LCh, light chain of FVIII; FVIIIa, activated FVIII; FX, factor X; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin; BU, Bethesda unit(s) determined by inhibitor assay.
The Factor VIII C2 Domain Binding to Factor Xa

Preparation of Anhydro-FXa

Anhydro-FXa, a catalytically inactive derivative of FXa in which dehydroalanine replaces the active-site serine, was prepared as described for the preparation of anhydrothrombin (36). In outline, FXa was chemically modified with phenylmethylsulfonyl fluoride (PMSF; Wako Pure Chemical Industries Ltd., Osaka, Japan). To convert the phenylmethylsulfonyl residues of the modified FXa to dehydroalanine residues, the product was diluted with 0.05 M NaOH to a final concentration of 12 mM NaOH in a 12 min at 0 °C, and the pH was adjusted to 7.5. After dialysis against 50 mM Tris-HCl, pH 7.5, containing 1 M NaCl, anhydro-FXa was purified by benzamidine-Sepharose 4B column chromatography (Amersham Pharmacia Biotech). The purified anhydro-FXa demonstrated <1% coagulant activity, and its molecular mass was estimated to be 43 kDa by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), similar to that of the native FXa (data not shown).

Synthetic Peptides

Synthetic peptides, each consisting of 13–18 overlapping amino acids and corresponding to the known epitope of monoclonal antibody ESH8 (residues 2248–2285), were synthesized by the method of simultaneous multiple peptide synthesis as described previously (37). They were analyzed and purified by reversed-phase high pressure liquid chromatography (purity >95%).

Activation of FXIII by FXa

FXIII (100 nM) was diluted in veronal buffer (50 mM sodium acetate, 7 mM sodium barbital, 0.1 M NaCl), containing 2% bovine serum albumin (BSA; Bovine Fraction V, Katayama Chemical, Osaka, Japan) and was incubated together with FXa (20 nM), PL vesicles (10 μM), and CaCl₂ (2.5 mM) at 37 °C. At timed intervals, samples (10 μl) were taken from the mixture, and FXa action was immediately quenched by 1000-fold dilution in 1 mM PMSF in veronal buffer at 4 °C. Each sample was tested for FXIII coagulant activity using a one-stage clotting assay. To assess the inhibitory effects of monoclonal antibodies on FXIII activation by FXa, each antibody was mixed with FXIII prior to FXa activation and incubated for 2 h at 37 °C. The anti-FXIII activity of each antibody was adjusted to 2 RU/ml. Control experiments indicated that the presence of FXa and PMSF in the diluted samples did not influence the FXIII activity during the coagulation assay.

Analyses of FXIII Cleavage by FXa

SDS-PAGE—125I-FXIII (10 nM) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, was mixed together with FXa (20 nM) and CaCl₂ (2.5 mM) in the presence or absence of PL vesicles (10 μM). The mixture was incubated at 37 °C for 30 min in the presence of PL and for 1 h in the absence of PL. At timed intervals, samples (20 μl) were taken, and FXa action was quenched by adding an equal volume of 0.4% SDS and immediately heating the samples to 100 °C for 5 min. Each sample was analyzed on 7.5% SDS-PAGE (38), followed by autoradiography of the dried gels. To assess the inhibitory effects of antibodies on FXIII cleavage by FXa, an equal volume of each antibody (1 μg) was mixed with 100 nM FXIII for 2 h at 37 °C prior to incubation with FXa, as described above.

To examine the cleavage of FXIII in the presence of vWF, 125I-FXIII (10 nM) was incubated with vWF (50 μg) for 1 h at 37 °C prior to the addition of FXIII in the absence of PL.

ELISA for Evaluation of FXa Cleavage of FXIII LCh—Microtiter wells (NUNC-Immuno Plate MaxiSorp, NUNC, Denmark) were coated overnight at 4 °C with 2 μg of each monoclonal antibody (NMC-VIII/5, ESH8, C5, or JR8) per well in 100 μl of coating buffer (0.1 M sodium bicarbonate, pH 9.6). After washing three times with washing buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20), the wells were then blocked for 2 h at 37 °C by the addition of coating buffer containing 4% BSA. After washing, FXIII (10 μg) in washing buffer containing 4% BSA was added to each well and incubated for 2 h at 37 °C. FXs (20 nM) and CaCl₂ (2.5 mM) were then added at 37 °C. At timed intervals, the supernatants were removed, and FXa action was quenched by the addition of 1 mM PMSF. Bound FXIII was detected by incubation with peroxidase-conjugated monoclonal antibody (NMC-VIII/5, ESH8, C5, or JR8) per well in 100 μl of horseradish peroxidase conjugate (0.05% hydrogen peroxide. After 2 min, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was added as a quenching solution, the absorbance was read at 492 nm (Lab system Multiskan Multisoft, Helsinki, Finland). Control experiments indicated that the presence of PMSF did not influence this system. Furthermore, SDS-PAGE confirmed that NMC-VIII/10, which was used to detect bound FXIII, did not itself inhibit FXa cleavage. The rate of FXIII LCh cleavage was calculated as follows: (1 – (bound – unbound)).

Table I: Properties of anti-FVIII monoclonal antibodies

| Antibody | anti-FVIII:C | domains bound in immuno blotting | Inhibitory effects on FVIII binding^a | vWF (IC₅₀) | PL (IC₅₀) |
|----------|--------------|---------------------------------|--------------------------------------|------------|------------|
| NMC-VIII/5 | 90           | C2                              | + (2.0)                              | (3.0)      | (3.0)      |
| ESH8     | 1,020         | + (2.0)                         | + (2.0)                              | (3.0)      | (3.0)      |
| NMC-VIII/10 | 24            | A3                              | + (5.0)                              | (3.0)      | (3.0)      |
| C5       | 450           | A1                              | –                                    | (3.0)      | (3.0)      |
| JR8      | 10,600        | A2                              | –                                    | (3.0)      | (3.0)      |

^a + and –, antibody inhibits or does not inhibit, respectively, FVIII binding to vWF or PL.

**Protein Indication**

Five μg of purified FVIII was radiolabeled by incubation with 0.5 mM of Na[125]I (Amersham Pharmacia Biotech) using IODO-GEN® (Pierce) for 3 min as described previously (35). Remaining free Na[125]I was removed by chromatography on a PD-10 column (Amersham Pharmacia Biotech). The specific radioactivity of 125I-FVIII was 10 μCi/μg protein. The activity of 125I-FVIII determined in a one-stage clotting assay was similar to that of unlabeled FVIII. Aliquots of radiolabeled FVIII were stored at ~80 °C for up to 1 month.

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nonspecific A492/bound at time zero – nonspecific A492) × 100(%). The absorbance reading in the absence of FVIII was regarded as nonspecific.

In competitive inhibition experiments using FVIII fragments (72-kDa LCh, A1, A2, or recombinant C2 domain), 2 μg of NMC-VIII/10 was coated onto microtiter wells as described above. In this assay, a 100 nM concentration of each FVIII fragment was added simultaneously with FXa prior to FXa action, and peroxidase-conjugated NMC-VIII/5 was used for detection of bound FVIII.

Measurement of Binding of FVIII to Immobilized Anhydro-FXa

Six μg of anhydro-FXa in 20 mM Tris-HCl, 150 mM NaCl (TBS), pH 7.4, were immobilized onto each well of a microtiter plate. After blocking with 4% BSA, serially diluted FVIII fragments in TBS, containing 2.5 mM CaCl2 and 4% BSA, were added and incubated for 2 h at 37 °C. Bound LCh or HCh fragment was detected by peroxidase-conjugated NMC-VIII/5 or JR8, respectively.

In competitive inhibition experiments using FVIII fragments, serially diluted 125I-FVIII was added to the immobilized anhydro-FXa. Bound 125I-FVIII was measured in a γ-counter. In this assay, serially diluted FVIII fragments were mixed with 125I-FVIII (100 nM) prior to adding to the anhydro-FXa. The percentage inhibition was calculated as follows: \(1 - \frac{\text{maximum bound at time zero} - \text{maximum nonspecific}}{\text{maximum nonspecific}} \times 100\%\). Radioactive counts in the absence of 125I-FVIII were regarded as nonspecific.

Kinetic Measurements Using Biomolecular Interaction Analysis

The kinetics of FVIII and anhydro-FXa interaction were determined by surface plasmon resonance using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). Anhydro-FXa was covalently bound to an activated carboxymethylated dextran-coated CM5 sensor chip surface by amine coupling according to the manufacturer’s recommendations (39, 40). Binding (association) of all ligands were monitored in TBS, pH 7.4, containing 2.5 mM CaCl2, 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 buffer flow. After each analysis, regeneration of the chip surface was achieved by 0.1 M glycine, pH 2.0, for 1 min. The values of equilibrium dissociation constants (\(K_d\)) and dissociation rate constants (\(k_d\)) were determined by nonlinear regression analysis as described previously (39, 40) using the evaluation software provided by Biacore AB. The values of equilibrium dissociation constants (\(K_d\)) were calculated as \(K_d = \frac{k_d}{k_a}\).

Inhibitory Effects of Synthetic Peptides on the Binding of the C2 Domain or LCh to Anhydro-FXa

Each overlapping peptide was serially diluted and mixed with 100 nM recombinant C2 domain or 72-kDa LCh fragment prior to addition to the immobilized anhydro-FXa. Bound FVIII was detected using peroxidase-conjugated NMC-VIII/5. Control experiments indicated that none of the synthetic peptides affected binding of FVIII fragments to NMC-VIII/5. The percentage inhibition was calculated as follows: \(1 - \frac{\text{maximum bound} - \text{nonspecific count}}{\text{maximum bound} - \text{nonspecific count}} \times 100\%\). Absorbance at 492 nm in the absence of FVIII was regarded as nonspecific.

RESULTS

Inhibitory Effects of Antibodies on FVIII Activation by FXa—Five min after incubation of FVIII with FXa there was an initial 4.5-fold increase in FVIII coagulant activity (Fig. 1A). Peak activity was followed by inactivation, and the base-line level was reached within 45 min of incubation. In order to examine the influence of anti-FVIII monoclonal antibodies, FXa-dependent FVIII activation in the presence of each antibody was performed in the presence of PL. Monoclonal antibody ESH8 recognizing the FVIII C2 domain strongly prevented the activation at a concentration of 2 BU/ml (Fig. 1A), and the inhibitory effect was dose-dependent (Fig. 1B). In contrast, the alternative anti-C2 monoclonal antibody, NMC-VIII/5, tended to enhance FVIII activation by FXa when compared with the pattern in the absence of antibody. None of the other monoclonal antibodies, anti-A1 antibody (C5), anti-A2 antibody (JR8), and anti-A3 antibody (NMC-VIII/10), inhibited FXa-dependent activation of FVIII at concentrations of 2 BU/ml (Fig. 1A) or 5 BU/ml (not shown).

Inhibitory Effects of Antibodies on FVIII Cleavage by FXa—We postulated that the inhibitory effect of the anti-C2 monoclonal antibody ESH8 on FVIII activation by FXa might be caused by either a change in the cleaved FVIIIa molecule that alters its coagulant activity or by a direct inhibition of the proteolytic cleavage of FVIII by FXa. To distinguish between these possibilities, we incubated 125I-labeled FVIII together with FXa for 1 h at 37 °C in the absence or presence of FVIII antibodies and then examined the cleavage pattern by SDS-PAGE. FVIII cleavage by FXa in the absence of antibodies resulted in the conversion of the 90–210 kDa fragments of the HCh into 54- and 44-kDa fragments and proteolysis of the 80-kDa fragment of the LCh into 72- and 67-kDa fragments (Fig. 2A, lane 2). In this instance, however, the 54-kDa fragment was observed only very weakly, and 47- and 40-kDa faint fragments as well as strong bands at dye front were also visible in the lower part of lane 2, suggesting that the 54-kDa fragment had been extensively proteolysed by FXa. ESH8 completely blocked the cleavage of the 80-kDa LCh fragment and reduced the formation of the 54- and 44-kDa fragments (Fig.

![Fig. 1](image_url)
FXα analyzed by SDS-PAGE. 125I-FVIII (10 nM) was incubated with
and JR8, respectively. Lanes 3–7 correspond to ESH8, NMC-VIII/5, NMC-VIII/10, C5, and JR8, respectively.

2A, lane 3). These results suggested that ESH8 completely prevented proteolytic cleavage at Arg1689 and Arg1721 in the LCh and partially inhibited Arg1772 in the HCh. In contrast, NMC-VIII/5 did not block the cleavage of the 80-kDa LCh fragment; rather, it tended to promote proteolysis, since the 72-kDa LCh fragment was more strongly evident than in other reactions (Fig. 2A, lane 4). Moreover, the 54-kDa fragment was markedly stronger and the 90-kDa fragment was not observed in the presence of NMC-VIII/5. These findings suggested that the antibody enhanced FXα-induced proteolysis of the HCh as well as the LCh, although selective inhibition of cleavage at Arg1689 by NMC-VIII/5 could not be excluded. NMC-VIII/10, C5, and JR8 did not interfere with FXα cleavage of FVIII (Fig. 2A, lanes 5–7, respectively).

The C2 domain contains a PL binding site, and several anti-C2 antibodies are known to inhibit proteolysis of PL by FXα (9). Furthermore, since FVIII cleavage by FXα occurs at a faster rate in the presence of PL than in its absence, inhibition of FVIII binding to PL results in indirect inhibition of FVIII cleavage by FXα. Therefore, we further examined the effects of monoclonal antibodies in the absence of PL. ESH8 again completely blocked the cleavage of the 80-kDa LCh fragment and delayed the formation of the 54- and 44-kDa fragments from the 90-kDa LCh fragment (Fig. 2B, lane 3). This finding indicated that the inhibitory effect of ESH8 was not due to the presence of PL. Also, the cleavages by FXα in the presence of NMC-VIII/5 in the non-PL system were similar to those obtained in the presence of PL and tended to be more marked in the presence of the antibody than in its absence (Fig. 2B, lane 4). Moreover, NMC-VIII/10, C5, and JR8 did not inhibit FVIII cleavage in the absence of PL (Fig. 2B, lanes 5–7, respectively). All of these findings indicated that the inhibitory effect of ESH8 on FXα-induced activation of FVIII could be attributed to a PL-independent mechanism, in particular inhibition of FVIII LCh proteolysis at Arg1689 and Arg1721.

Inhibitory Effects of vWF on FVIII Cleavage by FXα—We have previously reported that the monoclonal antibody NMC-VIII/5 promoted the dissociation of FVIII from the FVIII-vWF complex (11), whereas ESH8 prevented thrombin-induced dissociation of FVIII and vWF (14). We now observe from the above results that ESH8 and NMC-VIII/5 had opposite effects on FXα activation and cleavage of FVIII, although both recognize the C2 domain. Since the C2 domain is involved in FVIII binding to vWF, we further analyzed the effects of these two C2 monoclonal antibodies on FVIII cleavage by FXα in the presence of vWF by SDS-PAGE. vWF completely blocked the cleavage of 80-kDa LCh fragment and delayed the formation of 54- and 44-kDa fragments from the 90-kDa LCh fragment (Fig. 3, compare lane 3 with lane 2). These findings indicated that the vWF of the FVIII-vWF complex protected FVIII from the proteolytic activity of FXα. When ESH8 was added to FVIII in the presence of vWF prior to the addition of FXα, the protective effect of vWF remained evident, and as in the absence of antibody, cleavage of the LCh was completely inhibited, and proteolysis of the HCh was markedly delayed (Fig. 3, lane 4). In contrast, when NMC-VIII/5 was added prior to the addition of FXα, the protective effect of vWF was lost, and both the LCh and HCh were proteolyzed to their respective lower molecular weight fragments (Fig. 3, lane 5). These results were in keeping with our earlier findings that FVIII was dissociated from vWF by NMC-VIII/5 (11) and therefore became susceptible to proteolysis by FXα.

Inhibition of FXα Proteolysis of FVIII LCh by Monoclonal
Antibodies—In order to quantify the inhibitory effects of anti-FVIII antibodies on FVIII cleavage by FXa, we utilized an ELISA in the absence of PL. In this system, NMC-VIII/10 was used for detection, since it binds to the amino-terminal acidic region (epitope, 1675–1684) of LCh, does not bind to the cleaved 72- and 67-kDa LCh fragments, and does not interfere with FXa activity. It loses its binding ability if the LCh is cleaved at Arg1689 or Arg1721. The decrease in reactivity of NMC-VIII/10 with bound FVIII provides a measure, therefore, of FXa-dependent cleavage of the intact LCh. No cleavage of LCh was observed in the presence of ESH8 for 1 h after the addition of FXa (Fig. 4). In contrast, in the presence of NMC-VIII/5, C5, and JR8, cleavages of the LCh at Arg1689 and Arg1721 were almost complete (>95%) at 1 h. Notably, proteolysis of LCh by FXa in the presence of NMC-VIII/5 appeared to be more rapid than in the presence of C5 or JR8, thus confirming the results of SDS-PAGE analysis.

Competitive Inhibition of FXa Proteolysis of LCh by FVIII Fragments—Since the anti-C2 antibody ESH8 inhibited FXa-dependent proteolysis and activation, we investigated the hypothesis that the C2 domain is an essential domain for the association between FVIII and FXa. FVIII fragments (72-kDa LCh, A1, A2, or C2 domain) were tested for their ability to compete for the proteolysis of FVIII LCh by FXa in the ELISA described above. The 72-kDa LCh fragment competitively inhibited FXa cleavage of FVIII LCh by >95%. Similarly, the recombinant C2 domain inhibited cleavage by 63%. Minimal competitive inhibition (<5%) was observed, however, using the A1 and A2 domains (Fig. 5). These findings implicated a direct role for the C2 domain in FVIII and FXa association.

Binding of the C2 Domain to Immobilized Anhydro-FXa—To further investigate the relationship between the C2 domain and FXa, we developed an ELISA to measure the direct binding of FXa to FVIII fragments. To prevent the possible FXa-mediated cleavage of FVIII, we used active-site-modified FXa (anhydro-FXa), which lacks proteolytic activity. Binding was detected using NMC-VIII/5 or JR8 (known not to inhibit FXa action) for LCh or HCh, respectively. Control experiments showed that whole FVIII bound to immobilized anhydro-FXa in a dose-dependent manner (Fig. 6). Similarly, the 80- and 72-kDa LCh fragments demonstrated a dose-dependent binding pattern and appeared to bind more strongly than the whole FVIII (Fig. 6A). In addition, the C2 domain bound to anhydro-FXa in a dose-dependent manner, although in this instance the binding efficiency was approximately half that of the LCh fragments. The HCh fragment and the A2 domain showed little or no binding to anhydro-FXa (Fig. 6B).

Direct binding of FXa to FVIII was confirmed in a competitive assay using FVIII fragments and 125I-FVIII. The 80- and 72-kDa LCh fragments inhibited anhydro-FXa binding of 125I-FVIII by approximately 90%, and the C2 domain also inhibited binding by 58%. The HCh, however, did not inhibit binding of FVIII to anhydro-FXa (Fig. 7). These findings strongly suggested that the C2 domain is directly associated with the reactions between FVIII and FXa.

Kinetic Parameters of the Interaction between FVIII Fragments and Anhydro-FXa—The kinetic measurements (k_on, k_off, and K_d) for binding of FVIII to anhydro-FXa were calculated by surface plasmon resonance analysis and are illustrated in Table II. The K_d value for FVIII was 190 nM. The K_d values for the 80- and 72-kDa LCh fragments (55 and 51 nM, respectively) were approximately 4-fold less than that of the whole FVIII. The K_d value for the C2 domain was 560 nM. The HCh did not react with anhydro-FXa.

Inhibition of FVIII Fragment Binding to Anhydro-FXa by Synthetic Peptides—Since the anti-C2 monoclonal antibody,
ESH8, inhibited cleavage of FVIII by FXa and the recombinant C2 domain bound to anhydro-FXa, we focused on the known epitope structure of ESH8 (Fig. 8) to identify the potential FXa binding site. One of the synthetic peptides, designated EP-2 (residues 2253–2270), completely inhibited (>95%) binding of the recombinant C2 domain to anhydro-FXa. The concentration of the synthetic peptide producing 50% inhibition (IC50 value) was 13 μM (Fig. 9A). EP-2 also inhibited (84%) binding of the 72-kDa LCh fragment to anhydro-FXa (IC50 = 25 μM; Fig. 9B).

Similarly, the synthetic peptide EP-3 (residues 2258–2272) inhibited binding of the C2 domain and the 72-kDa LCh to anhydro-FXa but to a lesser extent (84 and 51%; IC50 = 25 and 100 μM, respectively). EP-4 (residues 2263–2277) partially inhibited binding to FXa, while EP-1 (residues 2248–2265), EP-5 (residues 2269–2281), and EP-6 (residues 2272–2285) demonstrated very weak or no inhibitory reactions. A control peptide (QHGDQISSILFEKVYMST) containing the same composition as EP-2 but in a random sequence did not interfere with the binding of FVIII fragments to anhydro-FXa binding (not shown). These findings indicated that the FXa binding site is located within residues 2253–2270 of the C2 domain of FVIII.

**DISCUSSION**

The present study revealed several novel findings that implicate the involvement of the C2 domain in the association between FVIII and FXa. First, the anti-C2 monoclonal antibody, ESH8, inhibited cleavage of FVIII by FXa, suggesting either that the antibody directly inhibited FXa binding to the C2 domain or that antibody binding produced a conformational change that prevented FXa cleavage. Second, the C2 domain as well as the 72-kDa LCh fragment of FVIII competitively inhibited FXa cleavage of the FVIII LCh. This finding indicated that the C2 domain is required for binding of FVIII to FXa rather than for cleavage of FVIII by FXa. Third, the C2 domain bound to a catalytically inactive FXa, anhydro-FXa, indicating more directly that the C2 domain contains the FXa binding site; and fourth, synthetic peptides, corresponding to sequences within the known epitope of ESH8, inhibited the binding of both the C2 domain and the 72-kDa LCh fragment of FVIII to anhydro-FXa. These data identified amino acid residues 2253–2270 within the C2 domain as essential for FXa binding.

In order to more precisely verify the relationship between the inhibitory effects of the monoclonal antibody and C2 epitope...
specificity, we compared ESH8 with another anti-C2 monoclonal antibody, NMC-VIII/5, which also inactivates FXIII. Although their anti-FVIII activities were comparably high, other characteristics were notably different. ESH8, which has an epitope within the region Val2248–Gly2285, strongly inhibited both activation and cleavage of FVIII by FXa but did not inhibit FVIII binding to PL (14). Conversely, NMC-VIII/5, which has an epitope within residues 2170–2327 and inhibits PL binding (11), enhanced the activation and cleavage of FVIII by FXa. These results suggested that the critical region for the association between FVIII and FXa is more likely to be within the amino-terminal C2 domain than in the PL binding region. Furthermore, the inhibitory effect of ESH8 on FVIII cleavage by FXa was not due to inhibition of FVIII binding to PL.

The cleavage of FVIII by FXa was completely inhibited by the addition of vWF in our PL-independent system. Similar inhibitory effects of vWF on FXa-dependent activation were previously reported by Koedam et al. (23). Those workers also described the loss of the amino-terminal 50-kDa HCh fragment in the absence of vWF, coincident with the formation of minor products of lower molecular mass, and suggested that the formation of the FVIII-vWF complex protects FVIII from extensive proteolytic cleavage by FXa. The formation of the FVIII-vWF complex also inhibits PL binding to FVIII. Because the vWF and PL binding sites have been identified in the C2 domain (8, 12), our results strongly support the involvement of the C2 domain in the association between FVIII and FXa. The earlier findings and our present data suggest that a vWF binding site is located close to the FXa binding site, although the possibility of steric hindrance of FXa due to conformational changes brought about when FVIII is complexed with vWF cannot be excluded.

The two anti-C2 monoclonal antibodies, ESH8 and NMC-VIII/5, show different characteristics in moderating FVIII and vWF interaction. ESH8 inhibits FVIII release from vWF (14), but NMC-VIII/5 dissociates FVIII from the FVIII-vWF complex and inhibits vWF binding (11). In the current investigation, NMC-VIII/5 did not inhibit the cleavage by FXa but rather tended to enhance it. Furthermore, the inhibitory effect of vWF on FXa-induced proteolysis of FVIII at both Arg1689 and Arg1721 was blocked in the presence of NMC-VIII/5 but was not blocked in the presence of ESH8. Thrombin cleaves FVIII only at Arg1689 in the LCh, and its reaction is not inhibited in the presence of vWF (23). It is evident, therefore, that activation of FVIII by FXa is regulated by the presence of vWF and that the binding mechanism for FXa is different from that of thrombin, although both proteases cleave at Arg1689.

Recently, a binding site for FX was localized within the acidic region of FVIII at the carboxyl terminus of the A1 domain (24). This was determined in binding experiments using immobilized FVIII captured by the same monoclonal antibody, ESH8, which inhibited FXa cleavage in our study. Those workers obtained similar results using FVIII immobilized on an anti-HCh monoclonal antibody and ruled out the involvement of the FVIII LCh in FX binding. We focused on FXa instead of FX, because FXII is proteolytically activated by FXa. It is not surprising that FXa binds to FVIII at a different site from FX. FXa binding sites in factor V, which is homologous with FVIII and has a similar domain structure, were recently identified within both the HCh and LCh (41). Furthermore, a membrane protein similar to the LCh of the factor V has been identified as a membrane receptor for FXa in leukocytes (42).

In order to demonstrate more directly that the C2 domain contains the FXa binding site, we used a catalytically inactivated derivative of FXa in our binding experiments. We prepared anhydro-FXa, in which the active-site serine was initially inactiv-ated by PMSF and then modified the phenylmethylsulfonyl serine to dehydroalanine by elimination of phenylmethylsulfonyl under alkaline conditions. Previous experiments using anhydrothrombin had shown that physiological substrate binding activity of the derivative is completely preserved under these conditions (36). In our investigation, the recombinant C2 domain or the LCh fragments (80 and 72 kDa) bound to anhydro-FXa in a dose-dependent and saturable manner. The $K_d$ value (560 nM) for the C2 domain was higher than those for the 80- and 72-kDa LCh fragments (55 and 51 nM, respectively). Several reasons for this lower affinity of the C2 domain can be considered. One explanation may be that at least the whole 72-kDa LCh is necessary for intact conformation of the C2 domain. The $K_d$ value for the binding to vWF of the C2 domain is similarly higher than that for the LCh (43). Since the $K_d$ value for the 80-kDa LCh fragment was very close to that for the 72-kDa LCh, it seemed unlikely that amino-terminal acidic region of the A3 domain was essential for FXa. It is pertinent that the HCh did not bind to anhydro-FXa. Furthermore, although proteolytic cleavage at Arg1721 in the HCh was inhibited by the anti-C2 monoclonal antibody ESH8, this inhibition was less than that seen at Arg1689 and Arg1721 in the LCh. These results suggest that the role of the C2 domain is more dominant than that of the HCh in FXa binding.

Finally, we obtained direct evidence for the presence of the FXa binding site in the C2 domain by competitive experiments using overlapping synthetic peptides based on the known epitope sequence of ESH8. One of the peptides (EP-2), corresponding to residues 2253–2270, completely (>95%) inhibited binding of the C2 domain and partially inhibited (84%) binding of the 72-kDa LCh fragment to anhydro-FXa. This inhibition was specific, since a control peptide, containing the same amino acids with a random sequence did not inhibit the reactions. An alternative peptide (EP-3), corresponding to residues 2258–2272, also inhibited binding of the C2 domain to anhydro-FXa. So it appears that residues KEFLISSSSQDGHQK2270 contained an important binding site for FXa in the C2 domain. These data provide strong evidence for the presence of a major binding site for FXa in the C2 domain of FVIII, although the possibility of the presence of an additional binding site in the A3–C1 region cannot be totally excluded.

Our findings provide the first direct evidence that the C2 domain of FVIII contains a major FXa binding site. Since FXa cleavage sites are located in the A3 domain, FXa may bind the C2 domain at a site remote from its active site. Our results also suggest that inhibition of FXa proteolytic activity may represent a new FXVIII inhibitory mechanism. Further studies are required to determine the precise physiological role of FXa binding to FVIII.

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