Replacing the Factor VIII C1 Domain with a Second C2 Domain Reduces Factor VIII Stability and Affinity for Factor IXa*

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Background: Factor VIII (FVIII), a plasma protein that is decreased or defective in individuals with hemophilia A, is expressed as both a heavy chain (HC) and a light chain (LC) composed of (a3)A3C1C2 domains. FVIII structure and intermolecular interactions result from alterations in stability and cofactor function. These results support an essential role for the C1 domain in membrane binding and functional properties of this variant as a cofactor for FIXa. Results from this study suggest that reductions in stability and cofactor function result from alterations in FVIII interdomain interactions and reduced affinity for FIXa. These results support an essential role for the C1 domain in FVIII structure and intermolecular interactions.

Materials—Recombinant FVIII (KogenateTM) and the monoclonal anti-A3 antibody 2D2 were generous gifts from Dr. Lisa Regan of Bayer Corp. (Berkeley, CA). Dioleoyl phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)) were purchased from Avanti Polar Lipids (Alabaster, AL). FVIII antibodies ESH4 (Sekisui Diagnostics, Stamford, CT), ESH8 (Sekisui Diagnostics), and GMA8011 (Green Mountain Antibody, Burlington, VT) were purchased from the indicated vendors. The reagents octadecyl rhodamine (OR) and 1-(2maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO-maleimide, 1-2-maleimidylethyl)-4-(5-(4-methoxyphenyl)-oxazol-2-yl)pyridinium methanesulfonate; PLV, phospholipid vesicle; APC, activated protein C.
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maleimide) (Invitrogen), α-thrombin, FVIIa, FIXaβ, FX, and FXa (Enzyme Research Laboratories, South Bend, IN), hirudin (DiaPharma, West Chester, OH), the chromogenic FXa substrate, Pefachrome Xa (Pef-a 5523, CH3OCO-D-CHA-Gly-Arg-pNA-AcOH; Centerchem Inc. Norwalk CT), and enhanced chemiluminescence reagent (GE Healthcare) were purchased from the indicated vendors.

Construction, Expression, and Purification of WT and Variant FVIII—WT FVIII and variants (FVIII C2C2) with C1 residues 2022–2168 replaced with C2 residues 2175–2325 were constructed as B-domainless FVIII, lacking residues Gln144–Ser1635 in the B-domain (14) (see Fig. 1A). Recombinant WT and variant FVIII forms were stably expressed in baby hamster kidney cells and purified as described previously (15). Protein yields for the variants ranged from >10 to ~10 μg from two 750-cm² culture flasks, with purity from ~85% to >95% as judged by SDS-PAGE. The primary contaminant in the FVIII preparations was albumin. FVIII concentration was measured by one-stage clotting and two-stage chromogenic FXa generation assays described below.

ELISA—A sandwich ELISA was performed as described previously (16) using purified recombinant FVIII (Kogenate, Bayer Corp.) as a standard. FVIII capture used the anti-C2 monoclonal antibody (GMA8011, ESH4, ESH8, or 2D2) for 2 min, and the activity was determined by one-stage cloting and two-stage chromogenic FXa generation assays described below.

One-stage Clotting Assay—One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII according a method as described previously (18) and assayed using a Diagnostica Stago clotting instrument.

FXa Generation Assay—The rate of conversion of FX to FXa was monitored in a purified system (19) according to methods described previously (20, 21). FVIII (1 nM) in 20 mM HEPES, 0.1 mM NaCl, 5 mM CaCl₂, 0.01% Tween 20, 0.01% BSA, pH 7.2 (HEPES buffer), containing 20 μM PSSPCPE vesicles (PLV, PS:PC:PE = 3:2:5) was activated with 20 mM α-thrombin for 1 min. The reaction was stopped by adding hirudin (10 units/ml), and the resulting FVIIIa was reacted with FIXa (40 nM) for 1 min. FX (300 nM) was added to initiate reactions, which were quenched after 1 min by the addition of 50 mM EDTA. FXa generated was determined following reaction with the chromogenic substrate Pefachrome Xa (0.46 mM final concentration). All reactions were run at 23 °C.

FXIII Thermal Decay—WT and FVIII C2C2 (4 nM) in HEPES buffer were incubated at 55 °C, aliquots were removed at the indicated time points, and activity was determined using the FXa generation assay.

FIXa Activity Decay—WT and FVIII C2C2 (1.5 nM) in HEPES buffer in the presence or absence of 20 μM PLV were activated using 20 nM thrombin for 1 min at 23 °C. Reactions were immediately quenched by hirudin (10 units/ml) to inactivate thrombin, aliquots removed at the indicated times, and activity was determined using the FXa generation assay.

FXIII Activity in the Presence of FVIII Antibody—WT and FVIII C2C2 (1 nM) in HEPES buffer in the presence or absence of 20 μM PLV were activated using 20 nM thrombin for 1 min at 23 °C followed by adding hirudin (10 units/ml) to inactivate thrombin. The reactions were incubated with 300 nM FVIII antibodies (GMA8011, ESH4, ESH8, or 2D2) for 2 min, and the activity was determined using the FXa generation assay.

Phospholipid Vesicle Preparation—Phospholipid vesicles containing OR (OR-PLV) were prepared by mixing 10 mg of PC:PE:PS and 0.6 mg of OR in 1 ml of chloroform and processed as described (22). This method yielded a concentration of 16.0 mM PC:PE:PS and 0.31 mM OR. OR concentration was determined by absorbance at 564 nm (molar extinction coefficient = 95,400). The number of OR molecules per unit of phospholipid area (Å²) was estimated to be 2.7 × 10⁻⁴ OR molecules/Å² based on the criterion that each phospholipid occupies an area of 70 Å² (22).

Fluorophore Labeling of FVIII—WT FVIII and FVIII C2C2 were labeled with PyMPO maleimide (excitation max/emission max = 417 nm/550 nm) as described (20) using a 10-fold molar excess of PyMPO maleimide over FVIII and incubated 4 h at 4 °C.

Phospholipid Binding of FVIII as Measured by Fluorescence Resonance Energy Transfer (FRET)—Titration of PyMPO maleimide-labeled WT FVIII or FVIII C2C2 was performed according to the methods described previously (8, 23). Briefly, 25 nM FVIII proteins (with or without PyMPO-labeling) in 20 mM HEPES, 0.1 mM NaCl, 0.01% Tween 20, 0.01% BSA, 5 mM CaCl₂, pH 7.2, containing 300 μM PC vesicles were titrated by adding 0–60 μM PLV or OR-PLV. Three separate titrations were performed including one where labeled FVIII was titrated with PLV without OR (sample-0), a labeled FVIII was titrated with PLV containing OR (sample-1), and an unlabeled FVIII was titrated with PLV with OR (sample-2). After the addition of PLV, samples were incubated for 10 min prior to determining emission fluorescence (540–546 nm; bandwidth 16 nm) by exciting at 417 nm (bandwidth: 2 nm) using an Aminco-Bowman Series 2 luminescence spectrometer (Thermo Spectronic). Actual fluorescence after quenching by OR (F) was calculated by subtracting sample-2 fluorescence from sample-1 fluorescence. Relative fluorescence (F/F₀), the ratio of F to control sample-0 fluorescence (F₀), was plotted against phospholipid concentration.

FIXa Binding Affinity—FVIII (0.5 nM) in HEPES buffer containing 20 μM PSSPCPE was activated by 20 nM thrombin for 1 min and immediately reacted with hirudin (10 units/ml), and

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the reaction was incubated in the absence or presence of each antibody (300 nM) for 2 min. Samples were then reacted with the indicated concentration of FIXa, and activity was measured by FXa generation assay.

*Michaelis-Menten Kinetics*—Thrombin-activated FVIII as described above (0.5 nM) in HEPES buffer containing 20 μM PSPCPE was incubated with 40 nM FIXa, and FXa generation was initiated by adding the indicated concentrations of FX. Data were fitted to the Michaelis-Menten equation by nonlinear least squares regression, and parameter values were obtained.

**FVIIIa Inactivation by Activated Protein C (APC) or FXa**—APC- or FXa-mediated inactivation of FVIIIa was performed as described previously (24, 25). Briefly, 150 nM FVIII was activated with 30 nM thrombin for 10 min at 37 °C. After mixing with 10 units/ml hirudin and 100 μM PLV, inactivation reactions were initiated by adding 3 nM APC or 5 nM FXa. Aliquots were removed at the indicated times, and residual FVIIIa activity was measured by a one-stage clotting assay.

**Data Analysis**—Values for FVIIIa activity decay as a function of time were fitted to a single exponential decay curve nonlinear least squares regression using the equation

\[
A = A_0 \times e^{-k \times t}
\]

where \(A\) is residual FVIIIa activity (nM/min/nM FVIII), \(A_0\) is the initial activity, \(k\) is the apparent rate constant, and \(t\) is time after FVIII activation when thrombin was quenched.

For FVIII-PLV binding kinetics, we used the following equation

\[
\frac{F/F_0}{A} = 1 - \frac{Q_{\text{max}}}{A} \times \frac{(A + K_d + X/n)^2 - \sqrt{(A + K_d + X/n)^2 - 4AX/n}}{2}
\]

where \(F/F_0\) is relative fluorescence, \(A\) is the concentration of FVIII (25 nM), \(X\) is the concentration of phospholipid, \(K_d\) is a dissociation constant, \(n\) is a ratio of binding stoichiometry (phospholipid:FVIII), and \(Q_{\text{max}}\) is the maximum quenching value. The value of \(n\) (= 100) was estimated as described previously (8).

FIXa-FVIII binding affinity used the following equation

\[
A = \frac{V_{\text{max}}}{B} \times \frac{(B + K_d + X)^2 - \sqrt{(B + K_d + X)^2 - 4 \times B \times X}}{2}
\]

where \(A\) is initial velocity (nM/min/nM FVIII), \(X\) is the concentration of FIXa in nM, \(K_d\) is the dissociation constant, \(B\) is the FVIIIa concentration, and \(V_{\text{max}}\) is the maximum activity at saturation.

We utilized a second order polynomial equation as employed previously for an unbiased estimation of the initial reaction rate (24).

\[
[FVIIIa] = A + Bt + Ct^2
\]

where \([FVIIIa]\) is the FVIIIa concentration in nM, \(t\) is the time in minutes, \(A\) is the initial concentration in nM of FVIIIa and 

\[
B\]

is the slope at time 0. Rates of FVIIIa inactivation were calculated by dividing the absolute value of \(B\) by the concentration of APC or FXa. Computation for nonlinear least squares regression analysis was performed using a standard curve-fitting algorithm (Gauss-Newton algorithm using the method of Levenberg-Marquardt).

**RESULTS**

**FVIIIC2C2 Sequence Structure, Western Blotting/Dot Blotting Analyses, and Cofactor Activity**—The domain construction of the FVIIIC2C2 variant is shown in Fig. 1. The C1 and C2 domains fold into nearly identical β-barrel structures (10, 11, 13). The corresponding region within the disulfide bridge of the C1 domain (residues Cys2021–Cys2169) was replaced by the homologous region in the C1 domain (residues 2022–2168) inside a disulfide bridge (*) with C2 domain (residues 2175–2325). B, left panel, following SDS-PAGE of FVIII (0.34 μg), proteins were transferred to PVDF membrane. Right panel, the indicated amounts of purified WT and FVIIIC2C2 proteins were transferred to PVDF membrane using a dot-blot apparatus and probed with 2D2 or GMA8011 antibody, and FVIII proteins were visualized by chemiluminescence as described under “Experimental Procedures.”
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TABLE 1
Specific activity of WT and FVIIIC2C2
Specific activity values were measured by one-stage clotting assay and FXa generation assay as described under “Experimental Procedures.” Data represent average values ± S.D. from three separate determinations. Values in parentheses are relative to the WT value.

|        | One-stage unit/µg | FXa generation (nm FXa/min/nM FVIII) |
|--------|-------------------|--------------------------------------|
| WT     | 4.60 ± 0.26 (1)   | 44.8 ± 1.6 (1)                       |
| FVIIIC2C2 | 0.72 ± 0.15 (0.16)| 15.8 ± 1.2 (0.35)                   |

TABLE 2
FVIII and FVIIa stability of WT and FVIIIC2C2
FVIII thermal decay at 55 °C and FVIIa spontaneous decay in the absence or presence of 20 µM PLV were measured as described under “Experimental Procedures” and plotted in Fig. 2. Data were fitted to a single exponential decay curve, and rate constant values ± S.D. were obtained. Values in parentheses are relative to the WT (in the absence of PLV) value.

| FVIII thermal decay rate constant (PLV present) | FVIIa spontaneous decay rate constant |
|-----------------------------------------------|-------------------------------------|
| WT                                           | PLV absent | PLV present |
| 0.047 ± 0.001 (1)                             | 0.157 ± 0.014 (1)                   | 0.091 ± 0.005 (0.6) |
| FVIIIC2C2 | 0.529 ± 0.057 (11.2) | 0.228 ± 0.023 (1.5) | 0.252 ± 0.023 (1.6) |

FIGURE 2. FVIII thermal stability at 55 °C (A) and FVIIa spontaneous decay (B). A, WT FVIII and FVIIIC2C2 (4 nM) were incubated at 55 °C, aliquots were taken at the indicated time points, and activity was measured by FXa generation assay as described under “Experimental Procedures.” B, thrombin-activated WT FVIIa (circles) and FVIIIC2C2 (triangles) (1.5 nM) were incubated at 23 °C in the absence (open symbols) or presence (closed symbols) of PLV (20 µM), aliquots were taken at the indicated time points, and activity was measured by FXa generation assay as described under “Experimental Procedures.” Each point represents a value averaged from three separate determinations. Data were fitted to a single exponential decay curve by nonlinear least squares regression, and dashed (in the absence of PLV) and solid (in the presence of PLV) lines were drawn.

FIGURE 3. Effects of anti-FVIII antibodies on FVIIa activity. Thrombin-activated FVIIa (1 nM) was incubated with the indicated anti-FVIII antibody (300 nM) for 2 min, and activity was measured by FXa generation assays as described under “Experimental Procedures.” FVIIa activity values expressed as blank (WT) or solid (FVIIIC2C2) bars represent the value averaged from three separate determinations, and standard deviations were drawn as lines on the top of the bars.

Stability of FVIII Variants—FVIII thermal decay at 55 °C as measured by FXa generation assay can be used to monitor the stability of intersubunit interaction (6). Fig. 2A shows results of the thermal decay at 55 °C for WT FVIII and FVIIIC2C2. Results obtained for WT FVIII showed ~80 and ~40% activity remaining after 5 and 18 min, respectively. However, the FVIIIC2C2 variant appeared significantly more labile with activity decaying to ~20% of the initial level in 5 min (Fig. 2A). The estimated decay rate for FVIIIC2C2 was 11.2-fold higher as compared with WT FVIII (Table 2). Although the interaction of FVIII and PLV at this elevated temperature is unclear, in the presence of PLV, the decay rate of WT FVIII was reduced by ~2-fold, whereas the decay rate of FVIIIC2C2 was essentially unchanged (data not shown).

FVIIa activity decay is governed by the dissociation of A2 subunit (26). Fig. 2B shows the results of FVIIa activity decay for WT FVIII and the FVIIIC2C2 variant. In the absence of PLV, FVIIa activity of both WT FVIII and FVIIIC2C2 decayed similarly, showing 40–50% activity at 4 min. In the presence of PLV, more FVIIa activity (~70%) was remaining after a 4-min incubation (Fig. 2B). However, FVIIIC2C2 activity was similarly reduced, showing ~40% activity after 4 min. In the absence of PLV, the FVIIa decay rate of FVIIIC2C2 was 1.5-fold greater than the WT value (Table 2). In the presence of PLV, the WT FVIIa decay rate was reduced by ~2-fold as compared with the
value measured in the absence of PLV, whereas the decay rate for FVIIIaC22 was independent of the presence of PLV.

FVIIIa Activity Inhibition by FVIII Antibodies—We examined FVIIIa cofactor activity in the presence of a panel of anti-FVIII monoclonal antibodies directed against domains in the FVIII LC (Fig. 3). ESH8 is known to bind a region in the FVIII C2 domain that overlaps a thrombin-binding site (27). Because we were interested in the effects of the antibodies on FVIIIa activity, we first activated FVIII with thrombin prior to reaction with the antibodies. However, due to the rapid decay of FVIIIa activity by A2 subunit dissociation, we limited the antibody incubation time to 2 min. Activity was measured by FXa generation assay. Under these conditions, the anti-A3 antibody (2D2) did not inhibit cofactor activity of either WT FVIII or FVIIIIC22.

On the other hand, the anti-C1 antibody (GMA8011) inhibited WT FVIII activity by ~40% but did not inhibit FVIIIIC22 activity. Two anti-C2 antibodies were evaluated. ESH8 showed little if any inhibition of WT FVIII, whereas ESH4 showed ~20% inhibition. However, both ESH8 and ESH4 inhibited the FVIIIIC22 variant to levels of ~50% and ~40%, respectively, of the original activity.

Binding of WT FVIII and FVIIIIC22 to PLV—To test whether the above FVIIIa activity inhibition by antibodies was due to altered PLV binding of FVIIIa, we measured PLV binding affinity in the presence of the antibodies. PLV titrations of WT FVIII and FVIIIIC22 were performed, and binding was detected by FRET using PyMPO-labeled FVIII (donor) and OR-PLV (acceptor) (Fig. 4). Relative fluorescence from PyMPO-labeled WT FVIII (Fig. 4A) as well as from FVIIIIC22 (Fig. 4B) decreased in a hyperbolic fashion as the concentration of OR-PLV was increased with saturation occurring at ~10 μM OR-PLV. With the exception of results obtained in the presence of ESH4, no significant changes in titration profiles were observed for either WT FVIII or FVIIIIC22. However, in the presence of ESH4, the extent of quenching due to OR-PLV binding was markedly reduced for both WT FVIII and FVIIIIC22, Kd values estimated for FVIIIIC22 binding to OR-PLV indicated a modestly reduced affinity (~2.8-fold) as compared with WT FVIII (Table 3). The FVIIIIC22 variant also showed a modest reduction in the extent of quenching at saturation as expressed by relative fluorescence (Fmax) of WT FVIII. In the presence of GMA8011, ESH8, or 2D2, the Kd values for WT FVIII binding to OR-PLV were slightly increased (1.9–2.8-fold), whereas a marked increase in the Kd value obtained in the presence of ESH4 was observed (~100-fold) that was accompanied by a significant increase (1.3-fold) in the Fmax value. Similarly, the Kd values for FVIIIIC22 binding to OR-PLV in the presence of GMA8011, ESH8, or 2D2 did not show significant differences from the value obtained in its absence, whereas the Kd value in the pres-
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Inhibiting ESH4 was significantly increased (7.6-fold), accompanied by an increase in the $F_{\text{max}}$ value (1.4-fold). Results obtained for WT FVIII with the ESH4 antibody are consistent with earlier studies showing that this anti-C2 antibody, but not ESH8, inhibits FVIII binding to phospholipid (9, 28, 29). Our results also show a similar specificity of inhibition for the FVIII$_{C2C2}$ variant. However, we note that the magnitude of PLV binding affinity reduction by ESH4 on FVIII$_{C2C2}$ was lower as compared with WT FVIII. Furthermore, the $F_{\text{max}}$ value for FVIII$_{C2C2}$ in the presence of ESH4 was significantly higher than that for WT FVIII. The reason(s) for these differential effects of ESH4 on WT FVIII and FVIII$_{C2C2}$ is not clear, but may reflect the fact that FVIII$_{C2C2}$ contains two binding sites for these antibodies.

$\text{FIX}a$ Binding Affinity—Overall, the impaired PLV binding due to the presence of ESH4 may only partially explain the reduction in FVIIIa activity inhibition by this antibody. Thus we further analyzed the effect of these antibodies on the FVIIIa-FIXa interaction. Functional affinity of WT FVIII and FVIII$_{C2C2}$ for FIXa was measured by titrating (thrombin-activated) FVIIIa (0.5 nM) with the indicated concentration of FIXa and assessing activity by FIXa generation assay. The reconstituted FXase activity values were plotted as a function of FIXa concentration, and results are shown in Fig. 5. FXase activity of WT FVIII and FVIII$_{C2C2}$ increased to a saturable level as FIXa concentration was increased. From the fitted curves, $K_d$ values (Table 4) for FVIII$_{C2C2}$ showed an 8.7-fold reduced affinity for FIXa as compared with WT FVIII. As shown in Fig. 5, GMA8011 markedly inhibited WT FVIIIa activity. This inhibition was not explained by impaired PLV binding because FXa generation reactions were run with excess (20 μM) PLV in the assay. Interestingly, in the presence of GMA8011, FIXa affinity for WT FVIII was markedly reduced (10.4-fold, Table 4). Although ESH4 significantly inhibited PLV binding of WT FVIII, this antibody did not alter FIXa binding affinity. Both ESH4 and ESH8 modestly (~4-fold) inhibited FIXa binding of FVIII$_{C2C2}$. None of antibodies showed inhibitory effects on either FXase complex with substrate FX (data not shown).

Michaelis-Menten Kinetics and FVIIIa Inactivation by APC or FXa—The structural integrity of the FVIII$_{C2C2}$ variant was assessed by additional functional experiments that included Michaelis-Menten kinetics and FVIIIa inactivation by both APC and FXa (Fig. 6). FXase activity with WT FVIII and FVIII$_{C2C2}$ titrated with FX showed hyperbolic curves that were saturable (Fig. 6A). Estimated $K_m$ values showed essentially no differences for the WT FVIII and FVIII$_{C2C2}$ (32.8 ± 2.6 and 26.3 ± 1.2 nM, respectively). Furthermore, both WT FVIII and FVIII$_{C2C2}$ were inactivated by APC or FXa nearly linearly with activity reduced by ~50% in ~12 min (Fig. 6B). Estimated inactivation rates were similar for APC inactivation rates for WT FVIII and FVIII$_{C2C2}$ (2.6 ± 0.14 and 2.97 ± 0.32 min$^{-1}$, respectively) and FXa inactivation rates for WT FVIII and

| TABLE 4  |
|-----------|
| **FIXa binding affinity of WT and FVIII$_{C2C2}$** |
| Initial velocity of FXa generation was measured as described under “Experimental Procedures” and plotted in Fig. 5 as a function of FIXa concentration. Data were fitted to the quadratic equation by nonlinear least squares regression, and dissociation constant values for FIXa were obtained. Data represent average values ± S.D. from three separate determinations. Values in parentheses are relative to control (without antibody). |
| FIXa binding affinity ($K_d$) | WT | FVIII$_{C2C2}$ |
|---|---|---|
| $\text{WT}$ | $\text{FVIII$_{C2C2}$}$ | $\text{WT}$ | $\text{FVIII$_{C2C2}$}$ |
| $\text{FIX}a$ | $\text{FIX}a$ | $\text{FIX}a$ | $\text{FIX}a$ |
| $\text{FVIII only}$ | $0.27 ± 0.01 (1)$ | $2.36 ± 0.07 (1)$ | $41.9 ± 0.34 (1)$ | $14.8 ± 0.19 (1)$ |
| + GMA8011 | $2.81 ± 0.17 (10.4)$ | $2.25 ± 0.19 (0.95)$ | $25.4 ± 1.01 (0.61)$ | $14.8 ± 0.34 (1)$ |
| + ESH4 | $0.24 ± 0.01 (0.89)$ | $8.69 ± 1.53 (3.68)$ | $33.9 ± 0.33 (0.81)$ | $7.7 ± 0.48 (0.52)$ |
| + ESH8 | $0.30 ± 0.03 (1.11)$ | $10.78 ± 0.92 (4.57)$ | $43.4 ± 0.83 (1.04)$ | $9.7 ± 0.32 (0.66)$ |
FVIII C2C2 (1.72 ± 0.13 and 1.63 ± 0.22 min⁻¹, respectively). These results showing similar functions of the FVIII C2C2 variant and WT as a cofactor in FXase as well as serving as a substrate for APC and FXa suggest that the C domains are properly folded in the variant.

**DISCUSSION**

In a previous study, we generated an FVIII variant lacking the C2 domain (ΔC2-FVIII) (8) and showed that this variant retained high affinity for PLV, thus providing evidence for the significant contribution of the C1 domain in PLV binding. Attempts to produce an analogous FVIII variant lacking the C1 domain have been unsuccessful for reasons that are not fully understood. Therefore, to gain insights into the role of the C domains in a variant lacking C1, we generated an FVIII variant, FVIIIC2C2, where C1 is now replaced with a second C2 domain. This variant showed several functional defects as compared with the WT protein. The FVIIIC2C2 variant retained low but appreciable cofactor activity. Similar to ΔC2-FVIII, the thermal stability of FVIIIC2C2 was dramatically reduced as compared with WT. Furthermore, whereas PLV binding affinity of FVIIIC2C2 was modestly lower (2.8-fold) than WT FVIII, the FIXa binding affinity was markedly reduced (8.7-fold).

FVIII C1 and C2 domains show 66.2% sequence homology (39.7% identity). A portion of these sequences is shown in Fig. 6. Phospholipid-binding sites have been identified in both domains (4–9). Because FVIIIC2C2 contains a duplicated C2 domain, it was not surprising that the magnitude of the reduction observed in PLV binding affinity as compared with WT FVIII (<3-fold) was less than that of ΔC2-FVIII (~14-fold) (8). However, FVIIIC2C2 had only half of the cofactor activity as compared with ΔC2-FVIII as measured in an FXa generation assay where PLV is saturating. This reduced activity likely derives in part from an altered interaction with FIXa, as well as reduced protein stability as a result of weakened interdomain interactions.

A number of studies indicate that FVIII stability as measured by thermal decay experiments depends heavily on the strength of FVIII interdomain interactions at A1–A2 or A2–A3 (30–32) and A1–C2 (33) interfaces. In addition, we recently reported that noncovalent interaction at the intrasubunit interface formed by A3 and C1 but not at the interface formed by C1 and C2 contributed to FVIII stability (34). In the FVIIIC2C2 variant, the interdomain interaction of A1 and C2 is maintained, however, the interaction originally between A3 and C1 is now weakened.

**FIGURE 6.** Michaelis-Menten analyses and FVIIIa inactivation by APC or FXa. A and B, Michaelis-Menten kinetics of FXase complex (A) and FVIIIa inactivation by APC (open symbols) or FXa (closed symbols) (B) of WT FVIII (circles) and FVIIIC2C2 (triangles) were analyzed by the methods as described under “Experimental Procedures.” Each point represents the value averaged from three separate determinations. Data were fitted to the Michaelis-Menten equation (A) or second order polynomial equation (B) by nonlinear least squares regression, and dashed (WT) and solid (FVIIIC2C2) lines were drawn. Control experiments showed that <10% inactivation of FVIIIa over the time course resulted from spontaneous decay due to A2 subunit dissociation.

**FIGURE 7.** Alignment of a portion of the FVIII C1 and C2 sequences. Protein sequence alignment data were obtained using the web tool UVa FASTA Server based on the Smith-Waterman method. Amino acids are indicated by the single-letter code. Binding epitopes for ESH4 (2303–2332) and ESH8 (2265–2280) in the C2 domain and the corresponding region in C1 are underlined. The A3-interactive region in C1 (residues 2114–2120) and the corresponding region in C2 are shown in a bold italic font.
between A3 and C2. Inasmuch as mutations that strengthen or weaken the A3-C1 interaction resulted in increased or decreased FVIII stability, respectively (34), we speculate that the non-native A3-C2 domain interaction in the variant is weaker than the native A3-C1 interaction, and this makes a significant contribution to the observed lability and potentially reduced specific activity of the FVIIIC2C2 variant. This contention is supported by comparison of the A3 domain-interactive sequence in the C1 domain, Thr2114–Thr2220, corresponding to C2 domain residues Leu2273–Asn2277 in FVIIIC2C2, which shows a low level 28.6% homology (14.3% identity, see Fig. 7).

In a recent study using fluorescence resonance energy transfer, we estimated distance values between multiple sites in FVIIla and the phospholipid membrane surface (23). Results from these distance calculations indicated that the molecular orientation of FVIIla bound the phospholipid membrane with a tilt angle of 30–50° rather than standing upright. This orientation combined with results from mutagenesis of selected A3 domain residues (Arg1719 and Arg1721) provided evidence that the A3 domain also interacted with the phospholipid membrane. The A3 domain possesses a prominent FIXa-interactive site (35), and a high affinity interaction has been observed for the A3C1C2 subunit of FVIIla (35, 36). The observation that the FVIIlaC2C2 variant showed a 10-fold reduced affinity for FIXa suggests a contribution of the C1 domain to this interaction.

Using the x-ray crystal structure of FVIII to model the FVIII-FIXa complex on PLV (11) suggests that the FVIII C1 domain resides on the membrane adjacent to FIXa. This result is consistent with the effects we observed with the anti-C1 antibody, GMA8011. Although this antibody did not inhibit the WT FVIII interaction with PLV, the antibody did inhibit membrane-dependent FVIIIa cofactor activity without affecting the $K_m$ for FX. Thus these results suggest that the antibody blocked the FVIIIa-FIXa interaction. In the case of FVIIIC2C2, the first C2 domain that replaces C1 would be adjacent to FIXa. This positioning explains the capacity of the two anti-C2 antibodies to reduce the affinity for FIXa of the variant FVIIla while showing no effect on the affinity of FIXa for WT FVIIIa.

Recently, Albert et al. (37) reported that the ESH8 epitope was restricted to within a relatively short sequence (Ser2265–Val2280) in the C2 domain. Based upon sequence alignments with C1 (Fig. 7), this region corresponds to C1 residues Ser2106–Leu2123, and these residues are thought to be in close proximity to an FIXa-interactive site based upon the FVIIIa-FIXa binding model (11). Interestingly, this C1 sequence region also contains an A3-interactive region (Thr2114–Thr2120). Therefore, the observed ESH8-dependent inhibition of cofactor activity may be combined with an inhibition of C2-A3 interaction in the variant. Furthermore, although the epitope for ESH4 is less well defined and maps to within C2 domain residues Thr2303–Tyr2332 (29), the corresponding region in C1 also appears to be in close proximity to FIXa in the modeled structure.

Results from the current study cannot distinguish whether the altered interaction of the FVIIIC2C2 variant with FIXa resulted from a direct interaction with FIXa following replacement of C1 with C2 or whether the reduced affinity was due to indirect effects caused by altered interdomain interactions between the A3-C domains. However, results from this study indicate that the FVIII C1 domain is likely located near FIXa in the FXase complex. Further studies are required to determine the existence of direct interaction between the FVIII C1 domain and FIXa.

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REFERENCES

1. Fay, P. J. (2004) Activation of factor VIII and mechanisms of cofactor action. Blood Rev. 18, 1–15
2. Gilbert, G. E., and Arena, A. A. (1996) Activation of the factor VIIIa-factor IXa enzyme complex of blood coagulation by membranes containing phosphatidylserine. J. Biol. Chem. 271, 11120–11125
3. Gilbert, G. E., and Drinkwater, D. (1993) Specific membrane binding of factor VIII is mediated by O-phospho-L-serine, a moiety of phosphatidyserine. Biochemistry 32, 9577–9585
4. Gilbert, G. E., Kaufman, R. J., Arena, A. A., Miao, H., and Pipe, S. W. (2002) Four hydrophobic amino acids of the factor VIII C2 domain are constituents of both the membrane-binding and von Willebrand factor-binding motifs. J. Biol. Chem. 277, 6374–6381
5. Lewis, D. A., Pound, M. L., and Ortel, T. L. (2003) Contributions of Asn1996, Met1999, and Phe2000 in the factor VIII C2 domain to cofactor activity, phospholipid-binding, and von Willebrand factor-binding. Thromb. Haemost. 89, 795–802
6. Meems, H., Meijer, A. B., Cullinan, D. B., Mertens, K., and Gilbert, G. E. (2009) Factor VIII C1 domain residues Lys2092 and Phe2093 contribute to membrane binding and cofactor activity. Blood 114, 3938–3946
7. Liu, Z., Lin, L., Yuan, C., Nicolaes, G. A., Chen, L., Meehan, E. J., Furie, B., Furie, B., and Huang, M. (2010) Trp2313–His2315 of factor VIII C2 domain is involved in membrane binding: structure of a complex between the C2 domain and an inhibitor of membrane binding. J. Biol. Chem. 285, 8824–8829
8. Wakabayashi, H., Griffiths, A. E., and Fay, P. J. (2010) Factor VIII lacking the C2 domain retains cofactor activity in vitro. J. Biol. Chem. 285, 25176–25184
9. Lü, J., Pipe, S. W., Miao, H., Iacquemin, M., and Gilbert, G. E. (2011) A membrane-interactive surface on the factor VIII C1 domain cooperates with the C2 domain for cofactor function. Blood 117, 3181–3189
10. Shen, B. W., Spiegel, P. C., Chang, C. H., Huh, J. W., Lee, J. S., Kim, J., Kim, Y. H., and Stoddard, B. L. (2008) The tertiary structure and domain organization of coagulation factor VIII. Blood 111, 1240–1247
11. Ngo, J. C., Huang, M., Roth, D. A., Furie, B. C., and Furie, B. (2008) Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. Structure 16, 597–606
12. Novakovic, V. A., Cullinan, D. B., Wakabayashi, H., Fay, P. J., Baleja, J. D., and Gilbert, G. E. (2011) Membrane-binding properties of the Factor VIII C2 domain. Biochem. J. 435, 187–196
13. Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K., and Stoddard, B. L. (1999) Structure of the C2 domain of human factor VIII at 1.5 Å resolution. Nature 402, 439–442
14. Doering, C., Parker, E. T., Healey, J. F., Craddock, H. N., Barrow, R. T., and Pete Lollar, P. (2002) Expression and characterization of recombinant murine factor VIII with C1 Replaced by C2
15. Wakabayashi, H., Su, Y. C., Ahmad, S. S., Walsh, P. N., and Fay, P. J. (2005) A Glu113Ala mutation within a factor VIII Ca2+-binding site enhances cofactor interactions in factor Xa. J. Biol. Chem. 280, 9577–9585
16. Wakabayashi, H., Freas, J., Zhou, Q., and Fay, P. J. (2004) Membrane-binding properties of the Factor VIII C2 domain. Structure 12, 597–606
17. Takeyama, M., Wakabayashi, H., and Fay, P. J. (2013) Contribution of factor VIII light-chain residues 2007–2016 to an activated protein C-interactive site. Thromb. Haemost. 109, 187–198
18. Over, J. (1984) Methodology of the one-stage assay of Factor VIII (VIII:C). *Scand. J. Haematol. Suppl.* 41, 13–24

19. Lollar, P., Fay, P. J., and Fass, D. N. (1993) Factor VIII and factor VIIIa. *Methods Enzymol.* 222, 128–143

20. Wakabayashi, H., Koszelak, M. E., Mastri, M., and Fay, P. J. (2001) Metal ion-independent association of factor VIII subunits and the roles of calcium and copper ions for cofactor activity and inter-subunit affinity. *Biochemistry* 40, 10293–10300

21. Wakabayashi, H., Schmidt, K. M., and Fay, P. J. (2002) Ca²⁺ binding to both the heavy and light chains of factor VIII is required for cofactor activity. *Biochemistry* 41, 8485–8492

22. Yegneswaran, S., Wood, G. M., Esmon, C. T., and Johnson, A. E. (1997) Protein S alters the active site location of activated protein C above the membrane surface: a fluorescence resonance energy transfer study of topography. *J. Biol. Chem.* 272, 25013–25021

23. Wakabayashi, H., and Fay, P. J. (2013) Molecular orientation of Factor VIIIa on the phospholipid membrane surface determined by fluorescence resonance energy transfer. *Biochem. J.* 456, 293–301

24. Varfaj, F., Neuberg, J., Jenkins, P. V., Wakabayashi, H., and Fay, P. J. (2006) Role of P1 residues Arg336 and Arg562 in the activated-Protein-C-catalysed inactivation of Factor VIIIa. *Biochem. J.* 396, 355–362

25. DeAngelis, J. P., Wakabayashi, H., and Fay, P. J. (2011) Decreasing hydrophobicity or disulfide bridging at the factor VIII A1 and C2 domain interface enhances procofactor stability. *Blood* 112, 25748–25755

26. Ansong, C., Miles, S. M., and Fay, P. J. (2006) Factor VIII A1 domain residues 97–105 represent a light chain-interactive site. *Biochemistry* 45, 13140–13149

27. Matsumoto, T., Nogami, K., Ogiwara, K., and Shima, M. (2012) A putative inhibitory mechanism in the tenase complex responsible for loss of coagulation function in acquired haemophilia A patients with anti-C2 autoantibodies. *Thromb. Haemost.* 107, 288–301

28. Scandella, D., Gilbert, G. E., Shima, M., Nakai, H., Eagleson, C., Felch, M., Prescott, R., Rajalakshmi, K. J., Hoyer, L. W., and Saenko, E. (1995) Some factor VIII inhibitor antibodies recognize a common epitope corresponding to C2 domain amino acids 2248 through 2312, which overlap a phospholipid-binding site. *Blood* 86, 1811–1819

29. Ahmad, S. S., and Walsh, P. N. (2005) Role of the C2 domain of factor VIIIa in the assembly of factor-X activating complex on the platelet membrane. *Biochemistry* 44, 13858–13865

30. Wakabayashi, H., and Fay, P. J. (2008) Identification of residues contributing to A2 domain-dependent structural stability in factor VIII and factor VIIIa. *J. Biol. Chem.* 283, 11645–11651

31. Wakabayashi, H., Varfaj, F., Deangelis, J., and Fay, P. J. (2008) Generation of enhanced stability factor VIII variants by replacement of charged residues at the A2 domain interface. *Blood* 112, 2761–2769

32. Wakabayashi, H., Griffiths, A. E., and Fay, P. J. (2009) Combining mutations of charged residues at the A2 domain interface enhances factor VIII stability over single point mutations. *J. Thromb. Haemost.* 7, 438–444

33. Wakabayashi, H., Griffiths, A. E., and Fay, P. J. (2011) Increasing hydrophobicity or disulfide bridging at the factor VIII A1 and C2 domain interface enhances procofactor stability. *J. Biol. Chem.* 286, 25748–25755

34. Wakabayashi, H., and Fay, P. J. (2013) Modification of Interdomain interfaces within the A3C1C2 subunit of factor VIII affects its stability and activity. *Blood* 112, 25748–25755

35. Scandella, D., Gilbert, G. E., Shima, M., Nakai, H., Eagleson, C., Felch, M., Prescott, R., Rajalakshmi, K. J., Hoyer, L. W., and Saenko, E. (1995) Some factor VIII inhibitor antibodies recognize a common epitope corresponding to C2 domain amino acids 2248 through 2312, which overlap a phospholipid-binding site. *Blood* 86, 1811–1819

36. Albert, T., Egler, C., Jakuschev, S., Schuldenzucker, U., Schmitt, A., Brokemper, O., Zabe-Kühn, M., Hoffmann, D., Oldenburg, J., and Schwaab, R. (2008) The B-cell epitope of the monoclonal anti-factor VIII antibody ESH8 characterized by peptide array analysis. *Thromb. Haemost.* 99, 634–637