Factor VIII Lacking the C2 Domain Retains Cofactor Activity in Vitro

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Factor (F) VIII consists of a heavy chain (A1A2B domains) and light chain (A3C1C2 domains). The activated form of FVIII, FVIIIa, functions as a cofactor for FIXa in catalyzing the membrane-dependent activation of FX. Whereas the FVIII C2 domain is believed to anchor FVIIIa to the phospholipid surface, recent x-ray crystal structures of FVIII suggest that the C1 domain may also contribute to this function. We constructed a FVIII variant lacking the C2 domain (designated ΔC2) to characterize the contributions of the C1 domain to function. Binding affinity of the ΔC2 variant to phospholipid vesicles as measured by energy transfer was reduced ~14-fold. However, the activity of ΔC2 as measured by FXa generation and one-stage clotting assays retained 76 and 36%, respectively, of the WT FVIII value. Modest reductions (~4-fold) were observed in the functional affinity of ΔC2 FVIII for FIXa and rates of thrombin activation. On the other hand, deletion of C2 resulted in significant reductions in FVIIIa stability (~3.6-fold). Thrombin generation assays showed peak thrombin and endogenous thrombin potential were reduced as much as ~60-fold. These effects likely result from a combination of the intermolecular functional defects plus reduced protein stability. Together, these results indicate that FVIII domains other than C2, likely C1, make significant contributions to membrane-binding and membrane-dependent function.

EXPERIMENTAL PROCEDURES

Materials—Recombinant FVIII (Kogenate™) and the monoclonal antibody 2D2 were generous gifts from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). Dioleoyl phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS)) were purchased from Avanti Polar Lipids (Alabaster, AL). Octadecylrhodamine (OR) and 1-(2-maleimidyldeyl)-4-(5-(4-methoxyphenyl)-oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide) were purchased from Invitrogen (Eugene, OR). The reagents α-thrombin, FVIIa, FIXa, and FXa (Enzyme Research Laboratories, South Bend, IN), hirudin (DiaPharma, West Chester, OH), and the chromogenic Xa substrate, Pefachrome Xa (Pefa-
5523, CH₃OCO-ᵳ-CHA-Gly-Arg-pNA:AcOH; Centerchem Inc. Norwalk, CT), recombinant human tissue factor (TF), Innovin (Dade Behring, Newark, DE), fluorogenic substrate, Z-Gly-Gly-Arg-AMC (Calbiochem, San Diego, CA), and thrombin calibrator (Diagnostica Stago, Parsippany, NJ) were purchased from the indicated vendors.

Construction, Expression, and Purification of WT and Variant FVIII—The FVIII variant composed of A1A2A3C1 domains was prepared as a B-domainless FVIII (lacking residues Gln744—Ser1637 in the B-domain (13)) using methods previously described (14). Recombinant WT and ΔC2 FVIII, the latter lacking >98% of the C2 domain (deletion of residues 2173–2332) were stably expressed in BHK cells and purified as described previously (14). Protein yields for the variants ranged 2173–2332) were stably expressed in BHK cells and purified as described previously (14). Protein yields for the variants ranged from >10 to ~100 μg from two 75 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 using an enzyme-linked immunoadsorbant assay (ELISA), and FVIII activity was determined by one-stage clotting and two-stage chromogenic FXa generation assays described below.

ELISA—A sandwich ELISA was performed as previously described (15) using purified recombinant FVIII (Kogenate, Bayer Corporation) as a standard. FVIII capture used the anti-A1 monoclonal antibody (GMA8002, Green Mountain Antibody) and the anti-A2 monoclonal antibody (R8812, Green Mountain Antibody) was employed for FVIII detection following its biotinylation.

SDS-PAGE and Western Blotting—FVIII proteins or FVIIIa proteins (activated by 20 nM thrombin for 30 min at 23 °C) (0.34 μg) were subjected to electrophoresis under reducing (0.1 M dithiothreitol) conditions using 10% polyacrylamide gels at constant voltage (150 V). Proteins were transferred to a polyvinylidene fluoride membrane, probed with an anti-A3 monoclonal antibody (2D2), and protein bands were visualized by chemifluorescence (570 nm) using a Storm 860 phosphorimager (GE Healthcare, Piscataway, NJ).

Phospholipid Vesicle Preparation—Phospholipid vesicles (large unilamellar vesicles) containing 20% PC, 50% PE, and 30% PS (or 100% PC) were prepared using octylglucoside as described previously (16). Phospholipid vesicles containing OR 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 (17). 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 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 (17).

Fluorophore Labeling of FVIII—WT and ΔC2 factor VIII were labeled with PyMPO maleimide (excitation max/emission max = 417 nm/550 nm) as described (18) using a 10-fold molar excess of PyMPO maleimide over FVIII and incubated 4 h at 4 °C. Labeled FVIII proteins (0.34 μg) were subjected to electrophoresis under reducing conditions using 10% polyacrylamide gels at constant voltage and transferred to a polyvinylidene fluoride membrane. Protein bands were visualized by Molecular Imager Gel Doc XR+ System. (Bio-Rad) by converting the UV light source to blue light with an Xcitablue Conversion Screen and detecting emission using the Qdots 525 mode.

Phospholipid Binding of FVIII as Measured by Fluorescence Energy Transfer—Titration of PyMPO maleimide-labeled WT or ΔC2 FVIII (25 nm) was performed by adding OR-containing phospholipid vesicles (0–15 μM for WT and 0–50 μM for ΔC2 FVIII) in the presence or absence of 500 nm WT (competitor) in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20, 0.01% BSA, 5 mM CaCl₂ (buffer A). In addition, 500 μM PC vesicles in the above buffer was added to reactions to prevent nonspecific binding. Binding was monitored by donor (PyMPO) emission quenching resulting from energy transfer to the acceptor (OR) as described (17). Briefly, 3 titrations were performed including one where labeled FVIII was titrated with phospholipid vesicles without OR (sample-0), a labeled FVIII titrated with phospholipid vesicles containing OR (sample-1), and an unlabeled FVIII titrated with phospholipid vesicles with OR (sample-2). After addition of phospholipid vesicles, 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, Rochester, NY). Actual fluorescence after quenching by OR (F) was calculated by subtracting sample-2 fluorescence (F₂) from sample-1 fluorescence (F₁). Relative fluorescence (F/F₀), which is the ratio of F to control sample-0 fluorescence (F₀) was plotted against phospholipid concentration.

One-stage Clotting Assay—One-stage clotting assays were performed using substrate plasma chemically depleted of FVIII (19) and assayed using a Diagnostica Stago clotting instrument. Plasma was incubated with APTT reagent (General Diagnostics) for 6 min at 37 °C after which a dilution of FVIII was added to the cuvette. After 1 min, the mixture was recalified, and clotting time was determined and compared with a pooled normal plasma standard.

FXa Generation Assay—The rate of conversion of FX to Fxa was monitored in a purified system (20) according to methods previously described (18, 21). FVIII (1 nM) in buffer A, containing 20 μM PSCPCE vesicles was activated with 20 nM α-thrombin for 10 s. The reaction was stopped by adding hirudin (10 units/ml) and the resulting FVIIa was reacted with FIXa (40 nM) for 10 s. FXa (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 μM final concentration). All reactions were run at 23 °C.

FVIIIa Activity Decay—WT and ΔC2 (1.5 nM) in buffer A containing 20 μM PSCPCE vesicles were activated using 20 nM thrombin for 10 s 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 following addition of FIXa (40 nM) and FX (300 nM).

Thrombin Generation Assay—The amount of thrombin generated in plasma was measured by Calibrated Automated Thrombography (22, 23). In a 96-well plate, 80 μl of FVIII-deficient plasma (<1% residual activity, platelet-poor) from a severe hemophilia A patient lacking FVIII inhibitor (George
King Bio-Medical, Overland Park, KS) was mixed with FVIII samples (20 μl, 6 nM) in HEPES-BSA buffer (20 mM HEPES, pH 7.35, 0.15 mM NaCl, 6% BSA) containing 6 pm TF (TF stock concentration was determined by Fx generation assay using known concentrations of FIXa), PSPCPF vesicles (2 μM) or 20 μl of thrombin calibrator (630 nM), and reactions were immediately started by mixing with 20 μl of fluorescent substrate (2.5 mM, Z-Gly-Gly-Arg-AMC) in HEPES-BSA buffer including 0.1 mM CaCl₂. All reagents were prewarmed at 37°C. Final concentrations of reagents were 0.25–32 nM FVIII, 1 pm TF, 4 μM PSPCPF vesicles, 433 μM fluorescent substrate, 13.3 mM CaCl₂, and 105 nM thrombin calibrator. The development of a fluorescent signal at 37°C was monitored at 8-s intervals using a Microplate Spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, CA) with a 355 nm (excitation)/460 nm (emission) filter set. Fluorescent signals were corrected by the reference signal from the thrombin calibrator samples (22), and actual thrombin generation in nM was calculated as previously described (23).

Michaelis-Menten Kinetics of FXase and Determination of FIXa Binding Affinity—FVIII (1 nM) in buffer A containing 10 μM PSPCPF vesicles was activated by 20 nM thrombin for 10 s, immediately reacted with hirudin (10 units/ml) and 40 nM FIXα, and FXα generation was initiated by adding the indicated concentrations of FX. For the measurement of FIXα binding affinity, FVIII (1 nM) in buffer A containing 20 μM PSPCPF was activated by 20 nM thrombin for 10 s, immediately reacted with hirudin (10 units/ml), and the indicated concentration of FIXα, and FXα generation was initiated by adding 300 nM FX as described above. Data were fitted to the Michaelis-Menten equation to determine kinetic parameter values or a quadratic equation to assess FIXα affinity by non-linear least squares regression.

Michaelis-Menten Kinetics of FVIII Activation by Thrombin—Various concentrations of FVIII (0–200 nM) in buffer A were activated by 0.5 nM thrombin for 10 s, and FVIIIα activity generated was determined by FXα generation assays. The concentration of FVIIIα was calculated from the FVIIIα activity value (250 nM/min and 190 nM/min for WT and ΔC2 FVIII) obtained when 1 nM FIXα was saturated with FVIIIα.

FVIII Cleavage by Thrombin—The kinetics of thrombin cleavage of FVIII was determined as described (24). Briefly, 200 nM FVIII in buffer A was activated by 2 nM thrombin for various times (0–30 min), quenched by SDS-PAGE buffer, and subjected to Western blot probed by R8B12 (anti-A2) and 2D2 (anti-A3) antibodies. The visualized band density was quantified by ImageQuant software (GE Healthcare). Because the total concentration of the A2-domain or A3-domain containing substrates and products totals 200 nM in each lane, the amounts of each species were calculated from a ratio of the band density value in each lane.

Data Analysis—For factor VIII-phospholipid binding kinetics we used Equation 1,

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

(Eq. 1)

where \(F/F_0\) is relative fluorescence, \(A\) is the concentration of factor VIII (25 nM), \(X\) is the concentration of phospholipid vesicles, \(K_d\) is a dissociation constant, \(n\) is a ratio of binding stoichiometry (phospholipid:factor VIII), and \(Q_{max}\) is the maximum quenching value. The value of \(n\) was estimated by titration using high concentrations of factor VIII (200 and 300 nM).

Based on the phospholipid concentration at the inflection points (20 μM for 200 nM factor VIII and 30 μM for 300 nM factor VIII titration), we determined that the value of \(n\) equals 100 (data not shown).

FVIIIα activity values as a function of time were fitted to a single exponential decay curve by non-linear least squares regression using Equation 2,

\[
A = A_0 \cdot e^{-kt}
\]

(Eq. 2)

where \(A\) is residual FVIIIα activity (nm/min/nM FVIII), \(A_0\) is the initial activity, \(k\) is the apparent rate constant, and \(t\) is the time after FVIII activation when thrombin was quenched. Michaelis-Menten kinetics using Equation 3,

\[
A = \frac{V_{max} \cdot X}{K_m + X}
\]

(Eq. 3)

where \(A\) is initial velocity (FXa generation in nm/min/nM FVIII or FXIII activation by thrombin in nm/min/nm thrombin), \(X\) is the concentration of enzyme substrate (FX or FVIII) in nM, \(K_m\) is the Michaelis constant, and \(V_{max}\) is the maximum activity at saturation. FXα-FVIII binding affinity used Equation 4,

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

(Eq. 4)

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

Thrombin cleavage data of FVIII as a function of time were fitted to a single exponential decay curve by non-linear least squares regression using Equation 5,

\[
A = A_0 \cdot e^{-kt}
\]

(Eq. 5)

where \(A\) is the amount of single chain FVIII (sfVIII in nm), \(A_0\) is the initial sFVIII (nm), \(k\) is the apparent rate constant, and \(t\) is the time (min), or Equation 6,

\[
A = A_0 (1 - e^{-kt})
\]

(Eq. 6)

where \(A\) is the amount of A2 or A3C1C2 (A3C1 in the case of ΔC2 FVIII) in nM, \(A_0\) is the initial subunit amount (nm), \(k\) is the apparent rate constant, and \(t\) is the time (min). Cleavage rates were calculated as \(A_0 \times k\).

Nonlinear least-squares regression analysis was performed by Kaleidagraph (Synergy, Reading, PA). For statistical analysis, a logarithmic scale parameter was used in place of the decay rate constant, and average values were compared by the Student’s t test.

RESULTS

Western Blot of the ΔC2 Mutant—The ΔC2 FVIII variant was constructed and expressed as described under “Experimental
Procedures.” Purified WT and ΔC2 FVIII were examined by Western blot before and after thrombin activation. As shown in Fig. 1, WT FVIII probed using an anti-A3 domain antibody (2D2), was visualized as a single chain FVIII (~170 kDa) and ~80 kDa light chain (lane 1). The ΔC2 single chain FVIII migrated at a slightly lower \( M_r \) position (~150 kDa, lane 2) compared with WT, and ΔC2 light chain migrated at ~60 kDa, consistent with deletion of the ~20 kDa C2 domain. After thrombin cleavage, the A3C1C2 subunit from WT FVIII migrated at ~70 kDa (lane 3), and the A3C1 band from ΔC2 FVIII appeared as an ~50-kDa band (lane 4). The light chain of WT FVIII appears as a doublet while that of ΔC2 LC appears as a triplet. The reason for this is not clear, although one of the triplet bands in ΔC2 LC (likely the highest \( M_r \) band) may be visualized because of the better resolution of the gel at the lower \( M_r \) position. The presence of residual uncleaved light chain seen in lane 4 may result from reduced thrombin cleavage efficiency of ΔC2 (see below).

Binding of WT and ΔC2 FVIII to Phospholipid Vesicles—PyMPO maleimide was incorporated into FVIII. Fig. 1 (lanes 5 and 6) show the PyMPO-labeled WT and ΔC2 FVIII, respectively, with bands detected by the bound fluorophore. Single chain FVIII (~170 kDa for WT and ~150 kDa for ΔC2), HC (~90 kDa), and LC (~80 kDa for WT and ~60 kDa for C2) bands were the predominant components accounting for >90% of the fluorescence in the FVIII preparations. The PyMPO fluorophor was selected as the fluorescence donor for the fluorescence acceptor, OR used to label the phospholipid vesicles, because the fluorescence overlap between donor emission (550 nm) and acceptor excitation (555 nm) maxima was satisfactory and the latter was far removed from the donor excitation maxima (417 nm). Results from phospholipid vesicle titration of WT and ΔC2 FVIII as detected by fluorescence energy transfer are shown in Fig. 2. Relative fluorescence from PyMPO-labeled WT as well as from the ΔC2 FVIII variant decreased in a hyperbolic fashion as the concentration of phospholipid containing OR increased. While fluorescence of the WT protein was reduced to ~0.7 at 5 μM phospholipid vesicles (open circles), ΔC2 fluorescence remained at ~0.95 (open triangles). The presence of a 20-fold excess of unlabeled WT FVIII (500 nM) during WT FVIII titration showed ~90 and ~70% reduced OR quenching at 5 and 15 μM OR phospholipid, respectively (closed circles). Similarly, in the case of ΔC2 FVIII titration the reduction of quenching in the presence of unlabeled WT FVIII (500 nM) was 70 and 50% at 5 and 15 μM, respectively (closed triangles). A \( K_d \) value for WT FVIII binding to OR-phospholipid vesicles was estimated as 3.2 ± 0.5 nM. The increase in ΔC2 fluorescence quenching showed a biphasic pattern with an inflection occurring at ~15 μM OR-phospholipid. Curve fitting for ΔC2 FVIII binding to OR-phospholipid using the initial portion of the curve (0–15 μM) yielded a \( K_d \) value of 43.6 ± 5.6 nM. This result suggested that the phospholipid binding affinity for ΔC2 FVIII was reduced by ~14-fold compared with WT. Titration of the FVIIIa WT and variant with OR-phospholipid in the presence of FXa (50 nM) resulted in no appreciable effect on membrane binding affinity (data not shown).

Cofactor Activity—ΔC2 FVIII showed 76% of WT specific activity as measured by a chromogenic FXa generation assay (Table 1) whereas the specific activity as measured by the one-stage clotting assay was more reduced (36% of WT activity). These results suggested that the reduced activity for the interaction of FVIII with phospholipid vesicles caused by C2 domain deletion still yielded a protein with appreciable cofactor activity under these assay conditions.

Thrombin Generation Profile of WT and ΔC2 FVIII—Thrombin generation parameter values were examined by calibrated thrombin generation assay using FVIII-deficient plasma. The

![FIGURE 1](image1.png)

**FIGURE 1. Western blot and fluorescence detection of WT and ΔC2 FVIII.** Purified WT (lanes 1 and 3) and ΔC2 FVIII proteins (lanes 2 and 4) (0.34 μg) were subjected to electrophoresis on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with 2D2 (anti-A3 domain antibody). Protein bands were visualized by chemiluminescence as described under “Experimental Procedures.” Lanes 1 and 2 show FVIII proteins before thrombin activation and lanes 3 and 4 show proteins after thrombin activation. The positions of the actual A3C1 bands of ΔC2 FVIII are indicated by dots on the right side. PyMPO-labeled WT (lane 3) and ΔC2 (lane 6) were subjected to electrophoresis on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and the fluorescence image was obtained by Molecular Imager Gel Doc XR+ System (Bio-Rad).

![FIGURE 2](image2.png)

**FIGURE 2. Binding of WT and ΔC2 FVIII to phospholipid vesicles detected by fluorescence energy transfer.** PyMPO-labeled WT (circles) and ΔC2 (triangles) FVIII (50 nM) in the presence (closed symbols) and absence (open symbols) of 500 nM WT FVIII in buffer A were titrated with phospholipid vesicles containing OR and emission at 540–545 nm was monitored as described under “Experimental Procedures.” \( F_0 \) is the fluorescence intensity of the sample titrated with unlabeled phospholipid. F is the corrected fluorescence intensity of the sample titrated with phospholipid vesicles containing OR. The acceptor density was \( 2.7 \times 10^{-9} \) OR molecules/Å². Data were fitted to an equilibrium binding equation by non-linear least squares regression as described under “Experimental Procedures.”
**C2 Domain-deleted Factor VIII**

**TABLE 1**
Specific activity and kinetic parameters for FXase complexes of WT and ΔC2 FVIII

Specific activity values were measured by one-stage clotting assay and FXa generation assay as described under "Experimental Procedures." The initial velocity of FXa generation was measured as described under "Experimental Procedures" and plotted in Fig. 4, A and B as a function of FX concentration and FIXa concentration, respectively. Data were fitted to the Michaelis-Menten equation or quadratic equation (for FIXa binding kinetics) by non-linear least squares regression, and the Michaelis constant (K_m) and dissociation constant for FIXa were obtained. Data represent average values and standard deviations from three separate determinations. Values in parentheses are relative to the WT value.

|            | Specific activity | Kinetic parameters |
|------------|-------------------|--------------------|
|            | Unit/μg | FXa Generation | Michaelis constant K_m | FIXa binding K_d |
|            | µM/FXa/min | FIXa generation | nM | nM |
| WT         | 4.53 ± 0.04 (1) | 49.9 ± 1.3 (1) | 15.7 ± 2.2 (1.0) | 0.27 ± 0.05 (1) |
| ΔC2        | 1.63 ± 0.06 (0.36)* | 37.9 ± 1.9 (0.76)* | 25.7 ± 2.1 (1.6)* | 1.25 ± 0.04 (4.5)* |

*p < 0.001.

*p < 0.05 compared to the value for WT.

**FIGURE 3. Thrombogram of FVIII proteins.** Thrombin generation assays in the presence of 1 pM TF and 4 μM PSCPCPE vesicles were performed according to "Experimental Procedures." Data shown are the estimated amounts of thrombin after the initiation of thrombin generation. Symbols and lines represent the values for WT FVIII (1 nM, open circles; 0.5 nM, black solid line; 0.25 nM, gray solid line) and ΔC2 FVIII (32 nM, black dotted line; 16 nM, gray dotted line).

**FIGURE 4. Functional properties of FVIII.** A, Michaelis-Menten analysis of the FXase complex. FVIII (1 nM) was activated by 20 nM thrombin for 10 s, immediately reacted with hirudin (10 unit/ml) and 40 nM FIXa, and FXa generation was initiated by adding the indicated concentrations of FX. Generated FXa was measured by FXa generation assay as described under "Experimental Procedures." B, measurement of functional K_d for FIXa and FXII association. FVIII (1 nM) was activated by 20 nM thrombin for 10 s, immediately reacted with hirudin (10 unit/ml) and the indicated concentration of FIXa, and FXa generation was initiated by adding 300 nM FX. Data were fitted to the quadratic equation by non-linear least squares regression and fit-lines were drawn. Each point represents the value averaged from three separate determinations. Values for WT (open circles) and ΔC2 (open triangles) are indicated.

reaction was initiated by 1 pM TF added in the presence of 4 μM PSCPCPE vesicles. After incubation of both WT and ΔC2 FVIII, an increase in thrombin generation was observed at ~5–7 min (Fig. 3) and reached the maximum at ~17–21 min. The peak thrombin and ETP (area under the curve) values obtained with 32 nM ΔC2 FVIII (62.9 nM and 1573 nM-min, respectively) were similar to those obtained using 0.5 nM WT FVIII (65.5 nM and 1558 nM-min). At a concentration of 16 nM ΔC2 FVIII, the peak and ETP values were similar to 0.25 nM WT FVIII (46.6 nM and 1196 nM-min for ΔC2 FVIII, and 41.6 nM and 1121 nM-min for WT FVIII). Therefore, we estimated that the thrombin generation potential of ΔC2 FVIII was reduced ~64-fold compared with WT FVIII. This result was more dramatic than the activity reductions observed using clotting and FXa generation assays. To investigate a potential reason(s) for this disparity we examined FXa generation kinetics, FIXa affinity, procofactor activation by thrombin and factor VIIIa stability of the ΔC2 FVIII.

**FIXa Binding Affinity of WT and ΔC2 FVIII**—The capacity of the ΔC2 FVIII mutants to serve as a cofactor for FIXa and the functional affinity for this interaction were examined by Michaelis-Menten kinetics. Results from the kinetics study (Fig. 4A) showed a saturable increase in activity as substrate FX concentration increases. The K_m value for reactions using ΔC2 FVIII estimated from non-linear least squares regression was increased by only ~1.6-fold as compared with the WT value (Table 1). Binding kinetics of FVIIIa with FIXa were examined by titrating FVIIIa (1 nM) after thrombin activation with various concentrations of FIXa (Fig. 4B). The hyperbolic increase in activity with increasing FIXa concentration was observed with near saturation achieved at 5 nM FIXa. The estimated affinity for FIXa, shown in Table 1, was moderately reduced as judged by an ~3.9-fold increase in apparent K_d as compared with WT.

**Thrombin Activation Kinetics and Cleavage Profile of WT and ΔC2 FVIII**—Michaelis-Menten analysis of FVIII activation by thrombin was performed by measuring the concentration of FVIIIa using FXa generation assays. Initial velocity of FVIIIa
generation was measured by the amount formed during a limited reaction time (10 s). As WT or ΔC2 FVIII substrate concentration increased, an increase in initial velocity was observed with saturation achieved at >200 nM (Fig. 5). Estimated $K_m$ values (Table 2) for both FVIII proteins were similar, with the ΔC2 FVIII variant showing a 30% increase in this parameter. However, the $V_{max}$ for ΔC2 FVIII was significantly lower (~2-fold, Table 2), suggesting some contribution of C2 domain to effectively orient the enzyme-substrate complex for efficient catalysis. Overall, conversion of the ΔC2 FVIII showed an ~2.8-fold reduction in catalytic efficiency ($V_{max}/K_m$) compared with the WT protein.

Cleavage rates for both FVIII proteins by thrombin were examined by Western blot and results are shown in Fig. 6A. Blots show the thrombin-activated FVIII samples at increasing time probed by R8B12 (a) and 2D2 (b) antibodies. WT FVIII protein appears as a single chain FVIII band (sFVIII), heavy chain (A1A2, R8B12 blot), light chain (a3A3C1C2, 2D2 blot), while the light chain of ΔC2 FVIII appears as a3A3C1 (2D2 blot) at the zero time point. The estimated subunit concentration during the time course of thrombin activation was plotted and results are shown in Fig. 6B (R8B12 blot) and 6C (2D2 blot). WT sFVIII disappeared within 5 min, while ΔC2 sFVIII disappeared more slowly with ~50% remaining at 5 min (Fig. 6B). Overall, the rate of loss of sFVIII appeared ~3.5-fold slower for the variant lacking C2 (Table 2). The appearance of A2 subunit in WT and ΔC2 FVIII proteins showed similar rates of increase, with a slightly slower rate for the ΔC2 FVIII substrate (Fig. 6B). This result suggested that cleavage at Arg$^{772}$ was not significantly affected by the lack of the C2 domain. In examining cleavage of the light chain at Arg$^{1689}$, the appearance of A3C1 from the ΔC2 FVIII variant was ~2-fold slower than that of A3C1C2 from the WT protein. Furthermore, the rate of cleavage at Arg$^{740}$ is likely reduced by as much as 2-fold in the ΔC2 FVIII based upon the rate of loss of single chain FVIII in the variant (~4-fold slower than WT), inasmuch as loss of this sub- strate is primarily governed by cleavages at Arg$^{740}$ and Arg$^{1689}$. Estimated cleavage rates are shown in Table 2. Overall, results from this analysis suggest a modest reduction in rates of proteolytic activation of FVIII when the C2 domain is deleted. This effect could contribute to the disparity in activities as measured in the one-stage clotting and Xa generation assays, which showed 36 and 76% the WT values, respectively. In the latter assay FVIII is fully activated using an excess of thrombin prior to formation of FXase and substrate addition, while in the former assay activation occurs in situ.

Stability of WT and ΔC2 FVIIIa—FVIIIa activity is labile because of A2 subunit dissociation after FVIII is activated by thrombin (25–27). Using the reaction conditions described under “Experimental Procedures” (1.5 nM FVIII) and as shown in Fig. 7, ~40% of WT FVIIIa activity was lost 4 min after activation by an excess of thrombin and only ~15% activity remained after 16 min. A decay rate of 0.128 ± 0.002 min$^{-1}$ for WT FVIIIa activity was determined. The FVIIIa activity decay rate was increased ~3.6-fold for the ΔC2 FVIII variant (0.466 ± 0.006 min$^{-1}$), which lost ~80% activity in 4 min. These results suggested that deletion of the C2 domain appears to significantly affect the stability of FVIIIa, which is in large part regulated by the rate at which A2 subunit dissociates.

**DISCUSSION**

A primary role ascribed to the FVIII C2 domain is binding phospholipid vesicles, which is a requisite step in assembly of FXase for the efficient conversion of FX to FXa during the propagation phase of coagulation. In this report we characterize a FVIII molecule lacking the C2 domain and show this variant retains significant cofactor activity as measured by in vitro assays. Results using a FXa generation assay where FVIII was pre-activated with an excess of thrombin, and reactions run with a high concentration of phospholipid vesicles and saturating substrate FX showed little difference between activity of the
WT and ΔC2 FVIII forms. Alternatively, a one-stage clotting assay showed the variant possessed about one-third the WT-like activity, whereas a thrombin generation assay using hemophilic plasma and initiated with a low concentration of TF revealed a ~60-fold reduction in activity of the ΔC2 FVIII variant relative to WT. The larger activity disparities observed in the latter assays possibly reflect defects in a variety of intermolecular interactions as well as cofactor stability. Furthermore, the large disparity in the thrombin generation parameters for WT and the ΔC2 FVIII variant relative to the other assays may also derive in part from the longer duration of this assay.

Early structure models of FVIII attributed phospholipid binding solely to the C2 domain with essentially no contribu-
tion from C1 (28). The intermediate resolution x-ray structures of FVIII (7, 8) show the C1 and C2 domains situated side-by-side beneath the three A domains and in an orientation where both C domains may interact with the phospholipid surface. Whereas the C2 domain appears essential for phospholipid binding (4), the FVIII structure strongly suggests that the C1 domain may also serve as an anchor for the phospholipid membrane surface. Recent evidence shows that recombinant FVIII C1C2 subunits were observed to bind more efficiently to platelets than the FVIII C2 subunit alone (10). In addition, a significant contribution of the C1 domain of FVa to the binding of the phospholipid surface was demonstrated by Ala-scanning mutagenesis (11, 12). A cluster of basic and hydrophobic residues in the FVIII C1 domain located opposite of the A domains (Trp2046, Ile2059, Trp2062, Lys2065, Arg2090, Lys2092, Phe2093, Leu2096, Val2130, His2155, Ile2158, and Arg2159) may be involved in this interaction. Recently, Meems et al. (29) reported the contribution of C1 domain residues Lys2092 and Phe2093 on membrane binding and activity as judged by mutagenesis studies.

Phospholipid vesicle binding affinity for WT ($K_d = 3.2 \text{ nm}$) was consistent with a reported value (4.8 nm) when vesicles were titrated with FVIII (30). Results in the present study show that the WT FVIII-phospholipid vesicle binding isotherm was similar to results from other studies where the binding was competed with phospholipid vesicles (30) or immobilized factor VIII was titrated with phospholipid vesicles (29). We observed an ~14-fold reduction in the binding affinity for the ΔC2 variant compared with the WT value. The $K_d$ for ΔC2-OR phospholipid vesicle interaction was 43.6 nm, which corresponded to a phospholipid concentration of 4.36 μM. Therefore, the reduction in phospholipid binding affinity observed for the ΔC2 variant did not likely affect the two-stage factor Xa generation assay value where excess (20 μM) phospholipid was present. The reason for the biphasic binding pattern of ΔC2 FVIII to vesicles is not clear. We speculate that the initial higher affinity binding likely reflected the physiologic state while deviations from this isotherm reflected lower affinity binding possibly resulting from nonspecific FVIII-OR phospholipid interactions.

Based upon these affinity values, free energy changes ($\Delta G$) at 23 °C for the WT and variant were calculated to be $-11.5 \text{ kcal/mol}$ and $-9.96 \text{ kcal}$, respectively. These data suggest a loss of ~13% of the binding energy with deletion of the C2 domain and implicate the C1 domain, either alone or in combination with other factor VIII domains, as providing significant contribution for membrane interaction.

In addition to its role in binding phospholipid membranes, the C2 domain has been implicated in other macromolecular binding interactions including those with VWF (3, 4), thrombin (5), and FIXa (6). Of these, the latter two could impact the functional assays described in this report. Nogami et al. (5) reported that C2 domain contains a thrombin binding site that may be particularly important for FVIII cleavage at Arg1689. Using an anti-C2 antibody, these investigators showed inhibition of Arg1689 cleavage as well as activity generation. Our results in the current study support this conclusion in that we observed rate reductions in cleavage at both Arg1689 and Arg740 as judged directly by cleavage of FVIII light chain and indirectly by the rate of loss of single chain FVIII. However, we speculate that this thrombin site is not a major interactive site given that overall rate of procofactor activation of the ΔC2 FVIII was reduced by only ~2-fold.

The FVIII A2 and A3 domains contain important sites for the interaction of the cofactor with FIXa (see Ref. (1) for review). Cross-linking studies suggest an interaction of the Glu domain of FIXa with the light chain of FVIII (6). Because the Glu domain is membrane-bound, these authors speculate that the interaction likely occurs at a site within the C2 domain. Our results suggest a possible contribution of the C2 domain to the affinity of FVIIIa for FIXa as judged by an ~4-fold increase in $K_d$ using the ΔC2 FVIII. However, as with the thrombin site in C2, this site may not be a predominant interactive region based upon the modest decrease in affinity. Interestingly, we observed a nearly 4-fold reduction in FVIIIa stability with the deletion of the C2 domain. This result was surprising given that the instability of FVIIIa reflects A2 subunit dissociation and the C2 domain does not directly contact A2. We speculate that a possible change around the membrane binding region of ΔC2 FVIII may allosterically affect A2 binding based upon observations that deletion of A1 residues 112 to 125 results in a similar fold reduction in FVIIIa stability. This altered cofactor stability, in combination with diminished intermolecular interactions for the ΔC2 variant may have contributed to observed reductions in thrombin generation parameters which are derived following a prolonged reaction time at 37 °C. We recently showed that mutations stabilizing the interaction of A2 subunit within FVIIIa resulted in increased parameter values such as peak thrombin and endogenous thrombin potential (31, 32), presumably due to enhanced duration of FXase activ-

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3 H. Wakabayashi and P. J. Fay, unpublished results.
ity. Thus the stability of FVIIIa, that appears to be impacted by the C2 domain, is a critical factor for efficient thrombin generation at plasma protein concentrations.

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