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Contribution of Factor VIII Light Chain Residues 2007–2016 to an Activated Protein C-Interactive Site

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Abstract

Although factor (F) VIIIa is inactivated by activated protein C (APC) through cleavages in the FVIII heavy chain-derived A1 (Arg³³⁶) and A2 subunits (Arg⁵⁶²), the FVIII light chain (LC) contributes to catalysis by binding the enzyme. ELISA-based binding assays showed that FVIII and FVIII LC bound to immobilized active site-modified activated protein C (DEGR-APC) (apparent K_d = 273 nM and 1.0 μ M, respectively). Furthermore, FVIII LC effectively competed with FVIIIa in blocking APC-catalyzed cleavage at Arg³³⁶ (K_i = 709 nM). A binding site previously identified near the C-terminal end of the A3 domain (residues 2007–2016) of FVIII LC was subjected to Ala-scanning mutagenesis. FXa generation assays and Western and dot blotting were employed to assess the contribution of these residues to FVIIIa interactions with APC. Virtually all variants tested showed some reductions in the rates of APC-catalyzed inactivation of the cofactor and cleavage at the primary inactivation site (Arg³³⁶), with maximal reductions in inactivation rates (~3-fold relative to WT) and cleavage rates (~3 to ~9-fold relative to WT) observed for the Met2010Ala, Ser2011Ala, and Leu2013Ala variants. Titration of FVIIIa substrate concentration monitoring cleavage by a dot blot assay indicated that these variants also showed ~3-fold increases relative to WT while a double mutant (Met2010Ala/Ser2011Ala) showed a >4-fold increase in K_m . These results show a contribution of a number of residues within the 2007–2016 sequence, and in particular residues Met2010, Ser2011, and Leu2013 to an APC-interactive site.

Keywords

factor VIIIa; activated protein C; factor VIII mutants; proteolysis

Introduction

FVIII¹ functions as a cofactor for FIXa in the enzyme complex (FXase) responsible for the anionic phospholipid surface-dependent conversion of FX to FXa (1). FVIII is synthesized as a multi-domain (A1-a1-A2-a2-B-a3-A3-C1-C2), single chain molecule consisting of 2332 amino acid residues with a molecular mass of ~300 kDa. This precursor is processed to a series of divalent metal ion-dependent heterodimers by cleavage at the B-A3 junction,

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Conflict of interest

None of the authors declare any conflict of interest.

¹Abbreviations: F, factor; LC, light chain; HC, heavy chain; VWF, von Willebrand factor; APC, activated protein C; DEGR, 1,5-dansyl-Glu-Gly-Arg; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; HBS, Hepes-buffered saline; CAPS, 3-(cyclo-hexylamino)-1-propanesulfonic acid; ELISA, enzyme-linked immunoadsorbent assay; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride

generating a HC consisting of the A1-a1-A2-a2-B domains and a LC consisting of the a3-A3-C1-C2 domains (2–4). Both single chain and heterodimer represent procofactor forms. The procofactor FVIII is activated by cleavage at Arg³⁷² (a1-A2 junction), Arg⁷⁴⁰ (a2-B junction), and Arg¹⁶⁸⁹ (a3-A3 junction) by thrombin or FXa, converting the dimer into the FVIIIa trimer composed of the A1, A2, and A3C1C2 subunits (5). The A1 and A3C1C2 subunits are stably associated while the A2 subunit remains associated through weak electrostatic interaction. Proteolysis at Arg³⁷² and Arg¹⁶⁸⁹ is essential for generating FVIIIa cofactor activity. Cleavage at the former site exposes a functional FIXa-interactive site within the A2 domain that is cryptic in the unactivated molecule (6). Cleavage at the latter site liberates the cofactor from its carrier protein, VWF (7) and contributes to the overall specific activity of cofactor (8).

Down-regulation of the FXase complex is due to inactivation of FVIIIa by two mechanisms (9). The first results from dissociation of the A2 subunit from the A1/A3-C1-C2 dimer (10), although the association of FVIIIa in the FXase complex partially stabilizes interactions with A2 and reduces the dissociation rate of this subunit (10). The second mechanism results from proteolytic inactivation and is catalyzed by activated protein C (APC) (5, 11) as well as FXa (5, 12). APC inactivates both FVIIIa and FVa, in membrane-dependent reactions (13). Inactivation of FVIIIa by APC results from cleaving the A1 subunit at Arg³³⁶ and A2 subunit at Arg⁵⁶² (11, 14). Reaction at the former site occurs at a significantly faster rate than at the latter site and closely correlates with loss of cofactor activity (11). APC binds within the A1 domain (15) and the LC subunit of the cofactors (16, 17). Earlier work from our laboratory identified an APC-binding site within the C-terminal end of the A3 domain (residues 2007–2016) of LC (18). However, APC-interactive residues within this sequence were not identified.

In this study we use a panel of point mutants where individual residues within the 2007–2016 sequence are individually replaced with Ala to assess potential contributions of each residue to APC-directed binding and catalysis. Results obtained from FXa generation and western and dot blotting assays suggest that a number of residues in this sequence contribute to these interactions with APC, with more prominent contributions attributed to Met2010, Ser2011 and Leu2013.

Materials and methods

Reagents

Recombinant FVIII (KogenateTM) and the monoclonal antibody 58.12 (19) recognizing the N-terminal end of the A1 domain were generous gifts from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). The monoclonal antibody C5 (20), which recognizes the C-terminal end of the A1 domain, was a gift from Dr. Zaverio Ruggeri. An anti-C2 monoclonal antibody (GMA-8003) was obtained from Green Mountain Antibodies (Burlington, VT). The reagents human α -thrombin, FXa, human APC, and human APC-DEGR (Hematologic Technologies Inc., Essex Junction, VT), FIXa and FX (Enzyme Research Laboratory, South Bend, IN), hirudin (DiaPharma, West Chester, OH), the chromogenic Xa substrate, Pefachrome Xa (Pefa-5523, CH₃OCO-D-CHA-Gly-Arg-pNAAcOH; Centerchem, Inc., Norwalk, CT), and TMB (3,3', 5,5'-tetramethylbenzidine) substrate (Thermo Fisher Scientific Inc., Rockford, IL) were purchased from the indicated vendors. Phospholipid vesicles (40% phosphatidylcholine (PC), 40% phosphatidylethanolamine (PE), and 20% phosphatidylserine (PS)) were prepared as described (21) using phospholipids purchased from Avanti Polar Lipids Inc. (Alabaster, AL).

Mutagenesis, Expression and Purification of Wild Type and Variant FVIII

Recombinant wild type FVIII, as well as FVIII variants His2007Ala, Met2010Ala, Ser2011Ala, Thr2012Ala, Leu2013Ala, Phe2014Ala, Leu2015Ala, Val2016Ala, and Met2010Ala/Ser2011Ala were constructed, expressed, and purified as described previously (22). Resultant FVIII forms were typically >90% pure as judged by SDS-PAGE with albumin representing the major contaminant. FVIII concentrations were measured using an enzyme-linked immunoadsorbent assay (ELISA) and FVIII activities were determined by one-stage clotting and a two-stage chromogenic FXa generation assays. FVIII samples were quick-frozen and stored at -80°C .

ELISA for Binding of FVIII to Immobilized DEGR-APC

Microtiter wells were coated with DEGR-APC (50 nM) in 20 mM Tris and 0.15 M NaCl, pH 7.4 (Tris-buffered saline; TBS), overnight at 4°C . The wells were washed with Hepes-buffered saline (HBS)-buffer (20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20) and were blocked with HBS-buffer containing 10% skim milk for 2 hours at room temperature. Various amounts of the FVIII were then added in HBS-buffer containing 5 mM CaCl_2 and 0.01% BSA for 2 hours at 37°C . Bound FVIII was quantified by the addition of biotinylated anti-FVIII C2 monoclonal antibody, GMA-8003 (10 $\mu\text{g/ml}$), followed by a streptavidin-peroxidase conjugated goat anti-mouse antibody (2 $\mu\text{g/ml}$) and TMB substrate. Reactions were quenched by the addition of an equal volume of 2 M H_2SO_4 , and absorbance values at 450 nm were measured using a microplate reader. The amount of non-specific binding of anti-FVIII C2 monoclonal antibody in the absence of DEGR-APC was <5% of the total signal. Specific binding was recorded after subtracting the non-specific binding.

FXa Generation Assay

The rate of conversion of FX to FXa was monitored in a purified system (23). FVIII (1 nM) in HBS-buffer containing 5 mM CaCl_2 and 100 μM PSPCPE vesicles was activated by the addition of thrombin (30 nM). Thrombin activity was inhibited after 1 min by the addition of hirudin (10 units/ml), and the resultant FVIIIa was reacted with APC (3 nM). Aliquots of 1 μl were removed at the indicated times to assess residual FVIIIa activity. FXa generation was initiated by the addition of FIXa (40 nM) and FX (300 nM) into the reaction mixture. The reactions were quenched after 1 min by adding 50 mM EDTA. Amounts of FXa generated were determined by addition of the chromogenic substrate Pefachrome Xa (0.46 mM final concentration) and rate of FXa generation were calculated. All reactions were run at 23°C . FVIIIa activity was determined as the amount of FXa generated per min and this value was used to determine the concentration of active FVIIIa.

Control experiments assessing FVIIIa stability were performed in the absence of APC in order to determine the rates of FVIIIa inactivation resulting from A2 subunit dissociation. At the concentrations of FVIIIa used in reactions, this value approximated an ~15 % loss of the initial activity for the 20 min time course. Therefore, for each time point in the time course experiments including APC, the obtained activity was corrected for the contribution of activity loss by the APC-independent mechanism.

Cleavage of FVIIIa by APC

APC (3 nM) was incubated with FVIIIa (130 nM) in HBS-buffer containing 5 mM CaCl_2 and PSPCPE (100 μM) at 37°C . Samples were taken at the indicated times, and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS-PAGE sample buffer and boiling for 3 min.

Electrophoresis and Western Blotting

SDS-PAGE was performed using 8% gels at 175 V for 50 min. For Western blotting, the proteins were transferred to a PVDF membrane at 100 V for 1 hour in buffer containing 10 mM CAPS [3-(cyclo-hexylamino)-1-pro-panesulfonic acid], pH 11 and 10% (v/v) methanol. Proteins were probed with anti-A1 domain monoclonal antibody (58.12), followed by goat anti-mouse alkaline phosphatase-linked second antibody. The signal was detected using the ECF system (Amersham Biosciences), and the blots were scanned at 570 nm using a Storm 860 instrument (Molecular Devices). Densitometric scans were quantitated using Image J 1.34 (National Institute of Health).

Dot Blotting

APC (3 nM) was incubated with various concentrations of FVIIIa (0–400 nM) in Hepes-buffered saline (HBS)-buffer (20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20) containing 5 mM CaCl₂ and PSPCPE (100 μM) at 37 °C. For competition studies, APC (3 nM) was incubated with FVIIIa (50 nM) in the presence of various concentrations of FVIII LC (0–2 μM) in the above buffer. Samples were taken at the indicated times, and the reactions were immediately terminated and prepared for dot blotting by boiling for 3 min. The samples were diluted with buffer containing 10 mM CAPS [3-(cyclo-hexylamino)-1-pro-panesulfonic acid], pH 11 and 10% (v/v) methanol and transferred to a PVDF membrane using Microfiltration blotting device (BIO-RAD, Hercules, CA). Proteins were probed with anti-A1 domain monoclonal antibody (C5), followed by goat anti-mouse alkaline phosphatase-linked second antibody. The signal was detected, and the blots were scanned as described above.

Data Analysis

All experiments were performed at least three separate times, and mean values and standard deviations are shown. Comparison of mean values was performed by the Student's t-test. Nonlinear least squares regression analyses were performed by Kaleidagraph (Synergy, Reading, PA). Studies of the interaction between DEGR-APC and FVIII measured by ELISA were performed using a single-site binding model by equation 1.

$$Absorbance = \frac{A_{\max} \cdot [S]}{K_d + [S]} \quad (\text{Eq. 1})$$

where [S] is the concentration of FVIII in the solid phase binding assay, K_d is the dissociation constant, and $A_{\max}$ represents maximum absorbance signal when the site is saturated by the FVIII.

Data from studies assessing FVIII LC-dependent inhibition of FVIIIa inactivation by APC were fitted by nonlinear least squares regression by using a competitive inhibition model,

$$\%binding = \frac{B_{\max} \cdot [L_0]}{K_d \cdot \left[1 + \frac{[L]}{K_i}\right] + [L_0]} + C \quad (\text{Eq. 2})$$

where L represents the concentration of FVIII LC, L_0 is the concentration of FVIII; $B_{\max}$ represents maximum binding, K_d is the dissociation constant for the interaction between FVIII and APC, K_i is the apparent inhibition constant for L , and C is a constant for binding of FVIII and DEGR-APC that was unaffected by L .

Analysis of FVIIIa cleavage by APC in the dot blot was performed using from curve fitting of a second order polynomial equation (Equation 3), as previously employed (24) to obtain slope values at time zero. Initial time points (up to 5 or 10 min) or up to approximately 40 % of substrate utilized were used for the analysis.

$$[FVIIIa] = A + Bt + Ct^2 \quad (\text{Eq. 3})$$

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 represents to the slope value at time zero. The absolute value of B is the rate of FVIIIa inactivation that was normalized by APC concentration. The results are expressed in nM FVIIIa/min/nM APC. Kinetic parameters were determined by dot blot analysis, monitoring time courses for cleavage of FVIIIa A1 subunit at Arg³³⁶. Values for K_m were calculated by fitting the data using a non-linear least-squares regression analysis to the Michaelis-Menten (Equation 4).

$$v = V_{max} \times [FVIIIa] / (K_m + [FVIIIa]) \quad (\text{Eq. 4})$$

where v is the initial velocity in nM, and $[FVIIIa]$ is the concentration of FVIIIa in nM.

Results

Specific activities of recombinant FVIII proteins

We previously demonstrated that FVIII LC contained an APC binding site (17) and residues 2007–2016 contributed to this binding interaction (18). This current study was undertaken to assess the contributions of individual residues within this sequence to binding to APC. For these studies, a series of recombinant FVIII proteins were prepared with individual residues within the 2007–2016 sequence replaced with Ala as previously described (22). Similar to wild type, SDS-PAGE of the purified variant proteins showed three bands of ~170, ~90, and ~80 kDa representing single chain FVIII, and HC and LC of the FVIII heterodimer, respectively, with overall purity of FVIII >90% (data not shown). Specific activity values measured for the FVIII mutant proteins were 45–74% as compared with wild type (Table 1), indicating a normal FVIII phenotype and suggesting that these residues were not critical to cofactor function of FVIIIa. Furthermore, the variants demonstrated that similar rates for activation by thrombin, as judged by the rates of FVIII cleavage and the generation of FVIIIa subunits, as compared with the wild type FVIII (results not shown). Taken together, these results suggested that point mutations with this sequence of LC did not affect overall FVIII conformation or function.

Interaction between FVIII (LC) and DEGR-APC

Earlier studies showed that the fluorescence of an eosin-labeled FVIII LC was quenched by DEGR-APC (17) indicating a direct binding interaction. However, the affinity of this interaction was not determined. An ELISA-based binding assay was employed to assess FVIII and FVIII LC binding to APC. Various concentrations of FVIII or FVIII LC were incubated with DEGR-APC (50 nM) that had been immobilized onto microtiter wells, and bound FVIII or FVIII LC was detected using a biotinylated anti-FVIII LC antibody, GMA-8003, as described in Materials and Methods. This antibody binds within the C2 domain of LC, a region not identified as interactive with APC and thus is not predicted to alter interaction of FVIII (LC) with DEGR-APC. FVIII (Figure 1A) and FVIII LC (Figure 1B) bound to DEGR-APC in a concentration-dependent and saturable manner. The data were well-fitted in a single-site binding model, yielding apparent K_d values of 273 ± 45 nM and 1.0 ± 0.2 μ M, for FVIII and LC, respectively. These results suggest the LC contains a

significant portion but not the complete interactive site and/or that the affinity of APC for LC is enhanced when LC is bound to HC due to a more favorable conformation.

In a complementary series of experiments, A1 subunit cleavage of FVIIIa at Arg³³⁶ by APC in the presence of various amount of FVIII LC was monitored using dot blotting as described in Materials and Methods. Using band density values of the A1 substrate, the initial velocity of A1 cleavage was estimated and plotted against FVIII LC concentration. These data were fitted to the nonlinear least-squares regression for a model of competitive inhibition using equation 2. We observed that FVIII LC inhibited the cleavage of FVIIIa by APC in a concentration-dependent manner (Figure 1B inset), with ~70% inhibition observed at the maximum concentration of FVIII LC employed (2 μ M). A K_i value of 710 ± 150 nM was estimated for FVIII LC and this value was similar to the estimated K_d for the LC-APC interaction.

Inactivation FVIIIa variants by APC

FVIII variants (150 nM) were activated by thrombin (30 nM). The resultant FVIIIa was reacted with 3 nM APC in the presence of 100 μ M PSPCPE vesicles. The reactions were then initiated with 300 nM FX and 40 nM FIXa, and cofactor activity was monitored for 20 min using a FXa generation assay as described in Materials and Methods (Figure 2). The values for cofactor inactivation rates were derived from the curve fits from the initial 5 min of the reactions. High concentrations of FVIIIa (150 nM) were used to minimize inactivation due to dissociation of A2 subunit from the A1/A3C1C2 dimer. The observed loss of FVIIIa activity obtained in the absence of APC was similar for the all variants and wild type FVIIIa forms (~10 % activity loss at 20 min, data not shown) and this value was used as a correction factor in determining APC-dependent inactivation rates. With the exception of the Phe2014Ala variant, which showed a similar rate of inactivation as the wild type FVIIIa, inactivation rates for the remaining variants were all reduced to a varying degree ranging from ~30 to ~70% the wild type value (Table 2). Three variants, Met2010Ala, Ser2011Ala and Leu2013Ala demonstrated similar and maximal (~3-fold) reductions in rate, suggesting these residues may make more prominent contribution to the interaction of enzyme with substrate than other residues within this sequence. On the basis of these observations, we prepared a double mutant (Met2010Ala/Ser2011Ala) to determine whether multiple mutations in this sequence yielded additive effects on inactivation rate. However, the inactivation rate for the Met2010Ala/Ser2011Ala variant FVIIIa substrate was reduced by a similar value (~3-fold) as the individual point mutations. These results suggest that a number of residues within the 2007–2016 make a moderate contribution to inactivation of FVIIIa by APC and that there may be an overall limit to this contribution in effecting reaction rate.

Cleavage of variants and wild type FVIIIa by APC

Western blotting was performed to monitor the rates of APC-catalyzed proteolysis at Arg³³⁶, the primary site for cleavage, and correlate this event with the reduced rates of FVIIIa inactivation. FVIIIa variants (130 nM) were incubated with APC (3 nM) and PSPCPE vesicles (100 μ M), and the reactions were quenched at specified intervals as described in Materials and Methods. Intact A1 (A1^{1–372}) and the product of proteolysis (A1^{1–336}) were visualized by Western blotting (Figure 3A) using a monoclonal antibody (58.12) that recognizes the N-terminal sequence of the A1 domain. Scanning densitometry obtained from the blots was employed to quantitate band densities of the substrate A1. Density values for A1 substrate and product were normalized to the FVIIIa concentration and non-linear least squares regression analysis was performed to calculate the rates of cleavage (Figure 3B). The values derived from curve fits were determined from the initial 5 min of the reactions.

Similar to the cofactor inactivation results, cleavage rates of A1 subunit at Arg³³⁶ for all variants were reduced to variable extents with the Met2010Ala, Ser2011Ala, and Leu2013Ala variants showing the greatest reductions (~10 to ~30% the wild type value) in rate (Table 2). Furthermore, the cleavage rate for Met2010Ala/Ser2011Ala variant paralleled that of the Met2010Ala variant showing an ~10-fold reduction in cleavage rate compared with wild type FVIIIa. Together, results from the cofactor inactivation and A1 cleavage experiments suggest that the FVIII sequence 2007–2016 contribute to the efficient inactivation of FVIIIa by APC with residues Met2010, Ser2011, and Leu2013 representing prominent determinants within this sequence for interaction with APC.

Michaelis–Menten analysis of A1 subunit cleavage using FVIII mutants

The above variants FVIII forms were evaluated in an A1 subunit cleavage assay using dot blotting where the FVIIIa substrate was titrated to assess K_m and k_{cat} parameters. This method was chosen to assess these parameter values rather than measurements relying on cofactor activity since low concentrations of the substrate FVIIIa show accelerated rates of A2 subunit dissociation yielding consequent loss of function (10). Thus this variable rate of loss of FVIIIa activity depending on its concentration and in the absence of APC precludes use of assays such as FXa generation. On the other hand, in FVIIIa the cleavage of Arg³³⁶ in the A1 subunit occurs independently of cleavage of Arg⁵⁶² in A2 subunit (25) and this rate is independent upon the presence of A2 subunit (11).

Cleavage of wild type FVIIIa (0–300 nM) and FVIIIa variants (0–400 nM) by APC (3 nM) in the presence of PSCPE (100 μ M) were monitored using dot blotting as described in Materials and Methods. This assay uses an anti-A1 subunit monoclonal antibody, C5, that reacts within the 337–372 C-terminal sequence of the A1 subunit that is cleaved by APC and released from A1^{1–336} as a 36-mer peptide fragment. Control experiments show that this cleavage fragment is not retained on the blot. Thus only intact (uncleaved) A1 subunit is visualized in the dot blot. Dot density values of the A1 substrate were used to estimate the initial velocity of A1 cleavage, and these data were plotted against FVIIIa concentration and fitted to the Michaelis-Menten equation. Results are presented in Figure 4 and summarized Table 3. With the exception of the Thr2012Ala variant, K_m values for all the variants tested were somewhat increased with the Met2010Ala, Ser2011Ala, and Leu2013Ala variants demonstrating the greatest increase (~2.5 to 3-fold) compared to wild type FVIII. Furthermore, the Met2010Ala/Ser2011Ala double mutant showed a somewhat greater increase (4.3-fold) in K_m compared to the wild type value than either individual point mutant. On the other hand, k_{cat} values were largely unaffected by the mutations with most values within ~40% of the wild type value. An exception was the double mutant which demonstrated an ~3-fold increase in k_{cat} . The reason for this is unclear but may result in part from a synergy of the increased k_{cat} values observed for both the individual point mutants. Overall, these kinetic data are consistent with results observed for binding in the absence of catalysis and indicate that the 2007–2016 sequence and in particular residues Met2010, Ser2011 and Leu2013 within this sequence contribute to an APC interactive site.

Discussion

Although APC does not catalyze cleavage within the FVIII LC (A3C1C2 subunit of FVIIIa), residues within this subunit are critical to efficient proteolytic inactivation of the cofactor. The present study was undertaken based upon two earlier observations. The first was demonstration that FVIII light chain contained an interactive site for APC as shown by dansyl-EGR APC quenching the fluorescence of an eosin-labeled FVIII LC (17). The second was that selected overlapping fragments of FVIII LC expressed in *E. coli* and containing the A3 domain 2007–2016 sequence, as well as a purified peptide prepared to this sequence, inhibited the inactivation of FVIII by APC (18). These earlier studies did not

provide estimates for the affinity of the inter-protein interaction or the contribution of FVIII residues 2007–2016 to an APC-binding site. Binding studies of FVIII and FVIII LC to immobilized DEGR-APC were performed using ELISA-based assays and yielded (apparent) K_d values 273 ± 45 nM and 1.0 ± 0.2 μ M, respectively. These values are apparent due to the non-equilibrium nature of the ELISA-based assay. It is important to note that this assay measures the inter-protein interaction in the absence of membranes and therefore do not identify any added benefit to the interaction that would be provided by phospholipid surfaces. However, the value of FVIII binding to immobilized DEGR-APC was similar to the K_m (102 nM) determined in a membrane-containing assay for FVIIIa inactivation by APC (25) adding validity to the binding assay employed.

Ala scanning mutagenesis over the FVIII 2007–2016 sequence revealed that a number of residues appear to make contributions to catalysis by APC affecting rates of FVIIIa inactivation and A1 subunit cleavage, and K_m . Of the residues tested, the Met2010Ala, Ser2011Ala and Leu2013Ala variants showed the most prominent effects (~3-fold to ~9-fold relative to WT) suggesting these residues may be more critical than others in interacting with the proteinase. Interestingly, a double mutation of Met2010Ala/Ser2011Ala showed essentially no further reductions in function relative to the single mutant showing the greater reduction, with the possible exception in K_m where the double mutant showed a slightly increased value compared with either point mutant. The reason for is unclear but suggests that either single mutation alone is sufficient to disrupt an interaction with APC that spans the two residues in the FVIII substrate.

Little structural information is available for the 2007–2016 sequence in FVIII. The intermediate resolution X-ray structures (26, 27) show that Met2010, Ser2011, and Thr2013 are exposed on the A3 surface. Comparisons of amino acid sequences of FVIII molecules (<http://europium.csc.mrc.ac.uk>) among human, porcine, murine, and canine indicate that these residues, except His2007 and Leu2013, are well-conserved, suggesting that this region may be fundamental for interaction with APC.

Substrate binding and cleavage of FVIII(a) by APC is driven by exosite interactions. Structural data have identified three surface loops, rich in basic residues, located in the protease domain of APC near the active site pocket (28). Mutations of these basic residues have been shown to variably inhibit the inactivation of FVIIIa by APC (15). These structures form anion-binding exosites that have been implicated in interacting with regions in FVIII rich in acidic residues such as residues 337–372 (25), which follow the primary cleavage site at Arg³³⁶ and include 13 acidic residues over the 36 residue long sequence. Unlike sites in the substrate interactive with the anion-binding exosites, the 2007–2016 sequence lacks any acidic residues to potentially form salt bridges with basic residues in APC. Thus other inter-protein interactions such as electrostatic and/or hydrophobic interactions are likely involved in this binding.

The 2007–2016 sequence is far removed from the primary bond cleaved by APC at Arg³³⁶ in the A1 subunit of the cofactor. Examination of the intermediate resolution X-ray structure of FVIII suggests a spatial separation of approximately 53 Angstroms. Thus we predict a similar distance separating the region in APC interacting with the 2007–2016 sequence and the proteinase active site. This sequence in FVIII lies at the C-terminal end of A3 close to the C1 domain. The C1 domain functions in membrane binding as judged by several lines of evidence including the observation that a C2 domain-deleted FVIII retains high affinity for the phospholipid surface (29). Furthermore, fluorescence resonance energy transfer studies indicate that the active site of APC is located high above the membrane surface (~94 Angstroms) (30), although accurate determination of this distance is made difficult by the low efficiency of transfer. Taken together, the large spatial separation of the binding and

active sites coupled with the membrane-proximal position of the interactive site in the FVIII substrate and membrane-distal position of the APC active site suggests that the complementary site in APC is contained within the Gla or EGF domains.

We recently showed that the 2007–2016 sequence also contains an interactive site of FX (22). Binding affinity determined for the two proteins as well as the K_m for FXase were most impacted by mutations at residues 2012, 2013 and 2014 indicating that the FX interactive site was not identical but rather overlapped with residues comprising the APC site. This observation for overlapping interactive sites for APC and FX was not surprising given the capacity for FX to protect FVIIIa from APC (31). In that study it was shown that inclusion of FX specifically blocked the APC-catalyzed cleavage of FVIIIa at Arg³³⁶ without affecting the rate of cleavage at Arg⁵⁶². Interestingly, the APC cofactor, protein S, abrogated this FX-protective effect by a mechanism that is not fully understood.

In conclusion, we demonstrate a role for selected residues in forming an APC-interactive site within the FVIII A3 domain sequence 2007–2016. A number of residues within this sequence, especially Met2010 and Ser2011 appear to contribute to the APC binding interaction and play a key role for FVIII inactivation by cleavage at Arg³³⁶.

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What is known about this topic?

- Factor (F) VIII functions as a cofactor for FIXa in the enzyme complex (FXase) and is responsible for the anionic phospholipid surface-dependent conversion of FX to FXa.
- Activated protein C (APC) inactivates FVIIIa in a membrane-dependent reaction down-regulating the coagulation system.
- APC binds within the A1 domain and the light chain (LC) of FVIII.

What does this paper add?

- Earlier work from our laboratory identified an APC-binding site within the C-terminal end of the A3 domain (residues 2007–2016) of LC. However, APC-interactive residues within this sequence were not identified.
- In this study, we use a panel of point mutants where individual residues within the 2007–2016 sequence are individually replaced with Ala to assess potential contributions of each residue to APC-directed binding and catalysis.
- Results obtained from FXa generation and western and dot blotting assays suggest that a number of residues in this sequence contribute to these interactions with APC, with more prominent contributions attributed to Met2010, Ser2011 and Leu2013.

Figure 1A

Figure 1B

Figure 1. Binding of FVIII and FVIII LC to APC

Binding of FVIII (Panel A) or FVIII LC (Panel B) to DEGR-APC by ELISA-based assays. Various concentrations of FVIII or FVIII LC were reacted with DEGR-APC (50 nM) immobilized onto microtiter wells. Bound FVIII or FVIII LC was detected using a biotinylated anti-FVIII C2 antibody, GMA-8003. Data were fitted using the Equation 1 (single-site binding model). Inset) Mixtures of FVIIIa (50 nM) and various concentrations of FVIII LC were incubated with APC (3 nM). Samples were taken at the indicated times and transferred to a PVDF. Proteins were probed with the anti-A1 domain monoclonal antibody (C5). The blots were scanned. Analysis of FVIIIa cleavage velocity by APC was performed using curve fitting for the second order polynomial equation (eq 3). Velocity value corresponding to FVIIIa cleavage by APC in the absence of competitor was defined as 100%. The data were fitted by non-linear least squares regression using eq 2 (competitive inhibition model). Experiments were performed at least three separate times and mean values are shown.

Figure 2A

Figure 2B

Figure 2. Inactivation of WT and FVIIIa mutants by APC

FVIII (150 nM) was activated by thrombin (30 nM). FVIIIa inactivation was then monitored over time in the presence of APC (3 nM) using a FXa generation assay. APC-catalyzed inactivation values were corrected by subtracting the corresponding values for FVIIIa decay obtained in the absence of APC. Solid lines were drawn from the curve fitting as described in Materials and Methods with dashed lines connecting points not used for determination of parameter values. The symbols used are as follows: wild type (open circles), His2007Ala (closed circles), Met2010Ala (open triangles), Ser2011Ala (closed triangles), The2012Ala (open squares), Leu2013Ala (closed squares), Phe2014Ala (open diamonds), Leu2015Ala (closed diamonds), Val2016Ala (crosses), and Met2010Ala/Ser2011Ala (asterisks). Experiments were performed at least three separate times, and mean values are shown.

Figure 3A

Figure 3B

Figure 3C

Figure 3. A1 subunit cleavage rates of the FVIIIa variants

(A) FVIIIa forms (130 nM) were activated by thrombin (30 nM) and then reacted with APC (3 nM). Aliquots were taken at indicated time points (0–30 min) and were subjected to SDS-PAGE. A1 substrate and A1^{1–336} product are visualized by western blotting using the 58.12 monoclonal antibody. (B and C) Loss of A1 subunit was calculated based on the band density values and plotted as a function of time for wild type. Lines were drawn from the curve fitting of second order polynomial equation (eq 3) as described under the Materials and Methods. The symbols used are as follows: wild type (open circles), His2007Ala (closed circles), Met2010Ala (open triangles), Ser2011Ala (closed triangles), The2012Ala (open squares), Leu2013Ala (closed squares), Phe2014Ala (open diamonds), Leu2015Ala (closed diamonds), Val2016Ala (crosses), and Met2010Ala/Ser2011Ala (asterisks). Experiments were performed at least three separate times, and typical results are shown.

Figure 4. Michaelis-Menten analysis of A1 subunit cleavage using FVIII mutants

Wild type FVIII (0–300 nM) and variants FVIII (0–400 nM) were activated by thrombin (30 nM) for 1 min in the presence of 100 μ M PSCPE and then reacted with 3 nM APC. Aliquots were taken at various time points (0–15 min) and were subjected to dot blotting. A1 subunit is visualized by C5 monoclonal antibody. Loss of A1 subunit was calculated based on the band density values and plotted. Analysis of A1 subunit cleavage velocity by APC was performed using a curve fitting of second order polynomial equation (eq 3). Data were fitted to Michaelis-Menten equation (eq 4) using non-linear regression analysis as described in the Materials and Methods. The symbols used are as follows: wild type (open circles), Met2010Ala (closed circles), Ser2011Ala (open squares), Leu2013Ala (closed squares), and Met2010Ala/Ser2011Ala (open triangles). Experiments were performed at least three separate times, and mean values are shown.

Table 1

Specific activity values for FVIII mutants

FVIII	Specific Activity
	%
WT	100 ± 7
His2007Ala	48.0 ± 1.9
Met2010Ala	52.8 ± 4.1
Ser2011Ala	60.8 ± 4.1
Thr2012Ala	62.7 ± 2.6
Leu2013Ala	55.1 ± 3.7
Phe2014Ala	72.4 ± 0.3
Leu2015Ala	50.8 ± 1.5
Val2016Ala	74.4 ± 1.7
Met2010Ala/Ser2011Ala	45.2 ± 1.7

Specific activity values are presented as a percentage and standard deviation of the wild type value, as described under Materials and Methods. Values are from at least three separate determinations.

Table 2Rates of FVIIIa inactivation and A1 subunit cleavage for wild type and mutant FVIII^a

FVIII form	Inactivation	A1 Cleavage
	<i>nM FVIIIa/min/nM APC</i>	<i>nM A1/min/nM APC</i>
WT	9.13 ± 0.53 (1.00)	8.80 ± 1.20 (1.00)
His2007Ala	4.40 ± 0.50 (0.48)	7.48 ± 1.04 (0.85)
Met2010Ala	3.10 ± 0.10 (0.34)	1.01 ± 0.43 (0.11)
Ser2011Ala	3.10 ± 0.47 (0.34)	1.81 ± 0.90 (0.21)
Thr2012Ala	6.21 ± 0.69 (0.68)	4.43 ± 0.41 (0.50)
Leu2013Ala	2.52 ± 0.22 (0.27)	2.69 ± 1.79 (0.30)
Phe2014Ala	8.92 ± 1.03 (0.98)	6.19 ± 0.39 (0.70)
Leu2015Ala	4.92 ± 1.35 (0.54)	6.55 ± 3.85 (0.74)
Val2016Ala	5.26 ± 0.11 (0.58)	4.43 ± 1.94 (0.50)
Met2010Ala/Ser2011Ala	2.83 ± 0.42 (0.31)	1.01 ± 0.12 (0.11)

Rates of FVIIIa inactivation and A1 cleavage were estimated by non-linear least-squares regression analysis from the data shown in Figure 1 and 2, respectively using Equation 1 as described under *Materials and Methods*. Data points represent mean ± standard deviations from at least three separate experiments.

Table 3Kinetic parameters for A1 cleavage of FVIII mutants by APC^a

FVIII form	K_m	k_{cat}
	<i>nM</i>	<i>min⁻¹</i>
WT	84.6 ± 3.7	5.6 ± 0.1
His2007Ala	133 ± 18 ^b	7.9 ± 0.5 ^c
Met2010Ala	214 ± 34 ^c	6.9 ± 0.5 ^b
Ser2011Ala	237 ± 64 ^b	8.6 ± 1.1 ^b
Thr2012Ala	57.8 ± 13.6	4.0 ± 0.3 ^c
Leu2013Ala	219 ± 33 ^c	9.0 ± 0.7 ^c
Phe2014Ala	105 ± 24	6.6 ± 0.6
Leu2015Ala	90.9 ± 26.9	5.2 ± 0.6
Val2016Ala	101 ± 6 ^b	5.9 ± 0.2
Met2010Ala/Ser2011Ala	368 ± 85 ^c	17.6 ± 2.3 ^c

^aReactions were performed as described under *Materials and Methods*. K_m and k_{cat} values ± standard deviations from three separate determinations were calculated by Michaelis-Menten kinetics.

^b $P < 0.05$ or

^c $P < 0.01$ compared with the value for wild type.