Sequences Flanking Arg\textsuperscript{336} in Factor VIIIa Modulate Factor Xa-catalyzed Cleavage Rates at this Site and Cofactor Function*

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Jennifer P. DeAngelis, Hironao Wakabayashi, and Philip J. Fay

From the Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642

**Background:** FXa initially activates FVIII and subsequently inactivates FVIII/FVIIIa by proteolysis at specific cleavage sites.

**Results:** Swapping sequences flanking these sites alters the rate by which FVIIIa is inactivated by FXa.

**Conclusion:** Sequences flanking cleavage sites contribute to the activation and inactivation of FVIII/FVIIIa.

**Significance:** This study suggests that flanking sequences modulate FVIII activation and inactivation pathways catalyzed by FXa.

Factor (F)VIII can be activated to FVIIIa by FXa following cleavages at Arg\textsuperscript{372}, Arg\textsuperscript{740}, and Arg\textsuperscript{1689}. FXa also cleaves FVIII/FVIIIa at Arg\textsuperscript{336} and Arg\textsuperscript{562} resulting in inactivation of the cofactor. These inactivating cleavages occur on a slower time scale than the activating ones. We assessed the contributions to cleavage rate and cofactor function of residues flanking Arg\textsuperscript{336}, the primary site yielding FVIII(a) inactivation, following replacement of these residues with those flanking the faster-reacting Arg\textsuperscript{740} and Arg\textsuperscript{372} sites and the slower-reacting Arg\textsuperscript{562} site. Replacing P4-P3' residues flanking Arg\textsuperscript{336} with those from Arg\textsuperscript{372} or Arg\textsuperscript{740} resulted in ~4–6-fold increases in rates of FXa-catalyzed inactivation of FVIIIa, which paralleled the rates of proteolysis at Arg\textsuperscript{336}. Examination of partial sequence replacements showed a predominant contribution of prime residues flanking the scissile bonds to the enhanced rates. Conversely, replacement of this sequence with residues flanking the slow-reacting Arg\textsuperscript{562} site yielded inactivation and cleavage rates that were ~40% that of the WT values. The capacity for FXa to activate FVIII variants where cleavage at Arg\textsuperscript{336} was accelerated due to flanking sequence replacement showed marked reductions in peak activity, whereas reducing the cleavage rate at this site enhanced peak activity. Furthermore, plasma-based thrombin generation assays employing the variants revealed significant reductions in multiple parameter values with acceleration of Arg\textsuperscript{336} cleavage suggesting increased down-regulation of FXase. Overall, these results are consistent with a model of competition for activating and inactivating cleavages catalyzed by FXa that is modulated in large part by sequences flanking the scissile bonds.

Factor VIII is an essential coagulation protein that when absent or defective, results in the bleeding disorder hemophilia A. Factor VIII circulates in the blood as an inactive procofactor. In heterologous cells, this procofactor is expressed in both single chain and heterodimer forms in near equivalence (see Ref. 1 for review). The single chain form is ordered as follows: A1-a1-A2-a2-B-a3-A3-C1-C2, with the three homologous A domains preceded or followed by acidic regions (designated by a lowercase a) (see Refs. 2 and 3 for review). The heterodimer is comprised of a heavy chain (A1-a1-A2-a2-B) and light chain (a3-A3-C1-C2) as result of post-translational processing at the B-a3 junction. The active cofactor form, factor VIIIa, is a heterotrimer consisting of A1, A2, and A3C1C2 subunits, generated by thrombin- or factor Xa-catalyzed cleavage at Arg\textsuperscript{372} (a1-A2 junction), Arg\textsuperscript{740} (a2-B junction), and Arg\textsuperscript{1689} (a2-A3 junction) (2). The A1 and A3C1C2 subunits retain the stable metal ion-dependent linkage, whereas the A2 domain is weakly associated in the trimer through electrostatic interactions (4–6). Factor VIIIa is a cofactor for the serine protease factor IXa, which together form the intrinsic factor Xase complex and catalyze the conversion of factor X to Xa during the propagation phase of coagulation (see Ref. 3 for review).

The factor Xase complex is down-regulated through two factor VIIIa-dependent mechanisms (see Ref. 3 for review). A non-proteolytic pathway results in factor VIIIa inactivation from the spontaneous dissociation of the A2 subunit from the A1/A3C1C2 dimer (7). The second mechanism, a proteolytic pathway, results from initial cleavage at Arg\textsuperscript{336} in the A1 subunit and subsequent cleavage at Arg\textsuperscript{562} in the A2 subunit, reactions that are catalyzed by both activated protein C (APC)\textsuperscript{2} (8, 9) and factor Xa (8, 10). Cleavage at the former site, which correlates with loss of function, alters orientation of the A2 subunit with the A1/A3C1C2 dimer (11), whereas cleavage at the latter site destroys a factor IXa-interactive site (12).

Factor Xa is both an activator (through cleavage at Arg\textsuperscript{372}, Arg\textsuperscript{740}, and Arg\textsuperscript{1689} (8)) and inactivator (through cleavage at Arg\textsuperscript{336} (8) and Arg\textsuperscript{562} (10)) of factor VIII(a). Proteolysis of Arg\textsuperscript{372} and Arg\textsuperscript{740} occurs at appreciably faster rates than cleavage at Arg\textsuperscript{336} (8, 13, 14) or Arg\textsuperscript{562} (10), and this property favors FXa as initially serving to activate factor VIII. We have previously shown that P4-P3' residues flanking the Arg\textsuperscript{336} and Arg\textsuperscript{562} sites contribute toward determining rates of APC cleav-

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1 To whom correspondence should be addressed: P. O. Box 712, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-275-6576; Fax: 585-275-6007; E-mail: philip_fay@urmc.rochester.edu.

2 The abbreviation used is: APC, activated protein C.
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age at these sites (15, 16). Given the homologies of APC and factor Xa as well as the common bonds cleaved during cofactor inactivation, we initiated a study to assess the roles of scissile bond flanking sequences in factor Xa-catalyzed inactivation of factor VIIIa. In this study, we examine the effects that replacing selected P4-P3’ residues flanking Arg336 with those flanking Arg372, Arg562, or Arg740 have on factor Xa-catalyzed cleavage and the regulation of cofactor activity. Results show variants possessing the Arg372 or Arg740 flanking sequence increase the and the regulation of cofactor activity. Results show variants possessing the Arg372 or Arg740 flanking sequences increase the factor Xa cleavage rate at Arg336 yielding increased inactivation rates and reduced peak activation levels. Conversely, either slowing cleavage at this site by replacement with the Arg562 flanking sequence or eliminating this cleavage by an R336Q mutation yielded increased cofactor activity and enhanced thrombin generation. These data indicate that sequences flanking the scissile bonds in factor VIII attacked by factor Xa make a significant contribution to proteolytic rates and influence activating/inactivating pathways catalyzed by this enzyme.

EXPERIMENTAL PROCEDURES

Materials—The 58.12 antibody, which recognizes the N-terminal sequence in the A1 domain, was provided by Bayer Corporation (Berkeley, CA). The RBB12 monoclonal antibody (GMA-012), which recognizes a discontinuous epitope within the A2 domain (17), and the GMA-8003 monoclonal antibody, which recognizes the C2 domain, were purchased from Green Mountain Antibodies (Burlington, VT). The B-domainless factor VIII expression vector (HSQ-MSAB-NotI-RENeo) and the Bluescript II K/S vector (20) were purchased from Green Mountain Antibodies. The B-domainless factor VIII expression vector (HSQ-MSAB-NotI-RENeo) and the Bluescript II K/S vector (20), and 100 mM phospholipid vesicles, 433 μM thrombin calibrator (Diagnostica Stago, Parsippany, NJ), were purchased from the indicated vendors. Phospholipid vesicles (20% phosphatidylserine, 40% phosphatidylcholine, 40% phosphatidylethanolamine; Avanti Polar Lipids Inc., Alabaster, AL) were prepared as described (18).

Construction, Expression, and Purification of Recombinant Factor VIII Mutants—Factor VIII proteins, 336(P4-P2)372, 336(P1’-P3’)372, and 336(P4-P3’)372 were made by mutating the specified P residues flanking Arg336 to those flanking Arg372, whereas 336(P4-P2)740, 336(P1’-P3’)740, and 336(P4-P3’)740 were made by mutating the specified P residues flanking Arg336 to those flanking Arg740. The factor VIII variants 336(P4-P3’)562 (15) and R336Q (19) were made by replacing P4-P3’ residues flanking Arg336 with those flanking Arg562 and by mutating Arg336 to Gln, respectively. Mutants were introduced into Bluescript II K/S—factor VIII cloning vector using the Stratagene QuikChange Site-directed Mutagenesis Kit and the mutated factor VIII coding region was ligated into the B-domainless factor VIII expression vector at XhoI/NotI restriction sites as described previously (20). Transfection and selection of the desired mutant proteins as well as protein expression in baby hamster kidney cells were performed as described previ-ously (20). Conditioned medium was collected daily and expressed proteins were purified using SP Sepharose (Amer sham Biosciences) as previously described (21). Active fractions were detected by a one-stage clotting assay, pooled, and dialyzed against 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 5 mM CaCl2, and 0.01% (v/v) Tween 20. Resultant factor VIII forms were typically >90% pure as determined by SDS-PAGE (8% gels).

ELISA—Concentrations of factor VIII proteins were determined by a sandwich ELISA using GMA-8003 as the capture antibody and biotinylated RBB12 as the detection antibody. The quantity of bound factor VIII was determined optically through use of streptavidin-linked horseradish peroxidase (Calbiochem, San Diego, CA) with o-phenylenediamine dihydrochloride (Sigma) as previously described (20). The standard used was the commercially purified factor VIII. ELISA and one-stage clotting assays were employed to determine specific activities of factor VIII proteins.

Activation of Factor VIII—Factor VIII (150 nM) was activated by thrombin (10 nM) in buffer containing 20 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM CaCl2, 0.1% (v/v) Tween 20, and 100 μg/ml BSA. After 5 min, thrombin activity was inhibited by addition of hirudin (20 units/ml) and the factor VIIIa product was reacted with factor Xa (3 nM) in the presence of phospholipid vesicles (100 μg/ml). Reactions were run at 22 °C. Aliquots were removed at the indicated times into buffer containing hirudin (20 units/ml) and activity was determined by a one-stage clotting assay as previously described (19).

Inactivation of Factor VIIIa by Factor Xa—Factor VIII (150 nM) was activated by thrombin (10 nM) in buffer containing 20 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM CaCl2, 0.1% (v/v) Tween 20, and 100 μg/ml BSA. After 5 min, thrombin activity was inhibited by addition of hirudin (20 units/ml) and the factor VIIIa product was reacted with factor Xa (3 nM) in the presence of phospholipid vesicles (100 μg/ml). Reactions were run at 22 °C. Aliquots were removed at the indicated time and assayed by one-stage clotting and Western blotting.

Western Blotting—Reactions were stopped at the indicated times with SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE using 8% polyacrylamide gels and Western blotting was performed as described previously (22). Blotting used the 58.12 (anti-A1) monoclonal antibody and binding was detected with a goat anti-mouse alkaline phosphatase-linked antibody (Sigma). The blots were developed using the ECF® (enhanced chemifluorescence) system (Amer sham Biosciences) and scanned at 570 nm using VersaDoc™ Imaging System (Bio-Rad). Band densities over a linear density range were measured using Image Lab (Bio-Rad).

Thrombin Generation Assay—The quantity of thrombin generated in plasma was determined through calibrated automated thrombography as previously described (23–25). Final concentrations of reagents used were 0.5–4 nM factor VIII (values for 1 nM are reported), 0.15 μM recombinant tissue factor, 4 μM phospholipid vesicles, 433 μM fluorogenic substrate, 13.3 mM CaCl2, and 105 nM thrombin calibrator. The fluorescent signal was monitored for 8-s intervals at 37 °C using a Microplate Spectrofluorometer (Spetramax Gemini, Molecular Devices, Sunnyvale, CA) at 355 nm (excitation)/460 nm (emission). A reference signal from the thrombin calibrator sample was used to correct fluorescent signals and actual thrombin generation in nanomolar was calculated as previously described (23).
RESULTS

Characterization of Recombinant Factor VIII Proteins—Factor VIIIa initiates activates factor VIII to factor VIIIa and subsequently inactivates the cofactor via proteolysis at specific scissile bonds. To determine the contributions of sequences flanking these bonds to the temporal nature of these processes, a series of recombinant B-domainless factor VIII mutant were prepared and stably expressed. Selected residues flanking the primary site for cofactor inactivation, at P1 residue Arg336, were replaced with those flanking the activating cleavage sites at the fast-reacting Arg740 and slower-reacting Arg372 sites (see Table 1). Additional variants were prepared where the sequence flanking a more secondary, slow-reacting inactivation site at P1 residue Arg562 was replaced with Arg336. Variants are designated based upon substituted flanking sequences. For example, the 336(P4-P2)740 variant has the P4-P2 residues flanking Arg372 (IQ); 336(P4-P2)372 variant replacing the original residues flanking Arg336 (PQL) to yield the P4-P3’ sequence: 333IQIRMKN339.

Specific activity values determined by one-stage clotting assay and ELISA for the purified factor VIII proteins were within 54–130% of the wild type factor VIII value (Table 2), suggesting that these mutations yield a normal phenotype and do not appreciably alter cofactor function. SDS-PAGE analysis of the proteins showed >90% purity, with typical bands of ~170 kDa for single chain factor VIII and bands of ~90 and ~80 kDa for the heavy and light chains of the heterodimer, respectively (data not shown). Furthermore, the activity for each variant was increased at least 15-fold by thrombin as judged by a one-stage clotting assay (Table 2). Overall, the increased factor VIIIa specific activity values of variants relative to wild type paralleled specific activity values for factor VIII. Furthermore, factor VIIIa decay rates for each variant were within a range of 0.11–0.16 min⁻¹ and similar to the wild type value of 0.13 min⁻¹ (data not shown) indicating that mutations in the sequences flanking Arg336 did not impact rates of A2 subunit dissociation.

Factor Xa-catalyzed Inactivation of P4-P3’ Factor VIIIa Variants—Factor VIII (150 nM) was activated to factor VIIIa by thrombin (10 nM) in a 5-min reaction, which resulted in near complete conversion of the procofactor to the active cofactor form (data not shown). Thrombin activity was quenched with hirudin (20 units/ml) and factor Xa was added (3 nM) in the presence of phospholipid (100 μg/ml), aliquots were removed at the indicated times, and activity was determined by one-stage clotting assay. Values are derived from curves fitted by nonlinear least squares regression analysis for the initial time points of the reactions and are corrected for the spontaneous decay of factor VIII observed in the absence of added factor Xa (Fig. 1). Rates for factor VIIIa inactivation resulting from factor Xa proteolysis are shown in Table 3. Replacing nonprime residues (P4-P2)247 with those flanking Arg336 resulted in an ~3-fold reduction in rates of inactivation, whereas replacement with prime residues (P1’-P3’) increased rates of inactivation by ~3-fold. The variant 336(P4-P3’)372, which combines these two mutations, resulted in an inactivation rate similar to that of wild type. Variants where the nonprime and prime residues flanking Arg336 were replaced with those flanking Arg562 resulted in no change and a slight increase in inactivation rates, respectively, whereas the complete flanking sequence variant 336(P4-P3’)740 exhibited an ~5-fold increased inactivation rate. The 336(P4-P3’)562 mutant exhibited an ~3-fold reduced rate of inactivation. Inactivation of the R336Q variant was markedly reduced (~12-fold reduction) compared with WT and this rate was attributed to slow cleavage at the Arg562 site.

Factor Xa-catalyzed Cleavage of P4-P3’ Variant Factor VIII A1 Subunit—Rates of factor Xa-catalyzed inactivation were correlated with cleavage of factor VIIIa at the primary site for inactivation, Arg336 in the A1 subunit, following SDS-PAGE and Western blotting. Blotting employed the monoclonal antibody 58.12, which recognizes the N terminus of the A1 subunit.

TABLE 1

| P4 | P3 | P2 | P1 | P1’ | P2’ | P3’ |
|----|----|----|----|-----|-----|-----|
| P  | Q  | L  | R336 | M   | K   | N   |
| V  | D  | Q  | R562 | G   | N   | Q   |
| I  | Q  | I  | R372 | S   | V   | A   |
| I  | E  | P  | R740 | S   | F   | S   |

*Sequences are indicated using the single letter amino acid designation.

**TABLE 2**

Specific activity values for P4-P3’ factor VIIIa variants

| Factor VIII | Specific activity | Activity following thrombin activation |
|-------------|-------------------|----------------------------------------|
| WT          | 4.5 ± 0.4 (1.0)*  | 120.0 ± 12.7 (1.0)                      |
| 336(P4-P2)372 | 2.6 ± 0.3 (0.6)  | 53.4 ± 4.4 (0.5)                        |
| 336(P1’-P3’)372 | 4.5 ± 0.5 (1.0)  | 75.4 ± 9.8 (0.6)                        |
| 336(P4-P3’)372 | 3.5 ± 0.2 (0.8)  | 64.2 ± 4.2 (0.5)                        |
| 336(P4-P2)740 | 5.8 ± 0.7 (1.3)  | 200.2 ± 31.8 (1.7)                      |
| 336(P1’-P3’)740 | 4.6 ± 0.4 (1.0)  | 75.1 ± 64.4 (0.6)                       |
| 336(P4-P3’)740 | 2.4 ± 0.2 (0.5)  | 57.5 ± 11.2 (0.5)                       |
| 336(P4-P3’)562 | 5.2 ± 0.3 (1.2)  | 142.1 ± 20.6 (1.2)                      |
| R336Q       | 3.8 ± 0.2 (0.8)  | 89.9 ± 5.3 (0.8)                        |

*Values in parentheses are activity values relative to wild type.

Data Analysis—All experiments shown were performed three or more times on separate occasions and the average values with standard deviations are shown. Analysis of Western blots was carried out by densitometry and nonlinear least squares regression analysis. Because the reaction model follows an integrated Michaelis-Menten equation rather than single exponential decay, we utilized a second-order polynomial equation (Equation 1) as previously employed (15) for an unbiased estimation of the initial reaction rate. This analysis used data from initial time points (up to 10 min) or up to ~40% of substrate consumed.

\[
[FVIIIa] = A + Bt + Ct^2
\] (Eq. 1)

Where \([FVIIIa]\) is the factor VIIIa concentration in nm, \(t\) is the time in minutes, \(A\) is the initial concentration in nm factor VIIIa or A1 subunit, and \(B\) is the slope at time 0. Rates of factor VIIIa inactivation, and A1 cleavage were calculated by dividing the absolute value of \(B\) by factor Xa concentration and results are expressed in nanomolar FVIIIa/min/nM factor Xa or nanomolar A1/min/nM factor Xa (15).
Modulation of Factor Xa Cleavage by Flanking Sequences

FIGURE 1. Factor Xa inactivation of the P4-P3′ factor VIIIa variants. Factor VIII (150 nM) was activated by thrombin (10 nM) for 5 min. Thrombin activity was quenched with hirudin (20 units/ml) and factor Xa was added (3 nM) in the presence of phospholipid (100 μg/ml), aliquots were removed at the indicated times, and activity was determined by a one-stage clotting assay as described under “Experimental Procedures.” Percent initial activity is plotted as a function of time for WT (○), 336(P4-P2)740 (△), 336(P1′-P3′)740 (●), 336(P4-P3)740 (×), 336(P4-P3)562 (□), and R336Q (△) proteins. Activity values were corrected for the spontaneous decay of factor VIIIa in the absence of added factor Xa, which accounted for an ~20% loss over the 30-min time course. Curve fits were applied for the initial time points (minimally 3 min, solid lines) and subsequent extended time points are shown with no lines. The values at 100% (starting activity following thrombin activation) were within ~900–3500 units/ml and values on the graph are presented as a percentage of this initial value. Experiments were performed at least three times with mean ± S.D. within 20%.

TABLE 3 Rates of factor Xa-catalyzed inactivation and A1 subunit cleavage of factor VIIIa variants

| Factor VIIIa | Factor VIIIa inactivation | A1 cleavage |
|--------------|---------------------------|-------------|
| nM FX VIIIa/min/nM FXa | nM A1/min/nM FXa |
| WT | 4.7 ± 0.7 (1.0)a | 5.4 ± 0.40 (1.0) |
| 336(P4-P2)372 | 1.5 ± 0.3 (0.3) | 1.0 ± 0.02 (0.2) |
| 336(P1′-P3′)372 | 12.5 ± 2.8 (2.7) | 22.0 ± 3.00 (4.1) |
| 336(P4-P3)372 | 6.2 ± 0.9 (1.3) | 9.3 ± 1.80 (1.7) |
| 336(P4-P2)740 | 4.6 ± 0.7 (1.0) | 8.3 ± 1.00 (1.5) |
| 336(P1′-P3′)740 | 7.6 ± 1.6 (1.6) | 15.6 ± 2.90 (2.9) |
| 336(P4-P3)740 | 25.2 ± 5.2 (5.4) | 50.2 ± 5.00 (5.6) |
| 336(P4-P3)562 | 1.7 ± 0.4 (0.4) | 2.2 ± 0.30 (0.4) |
| Arg336Gln | 0.4 ± 0.1 (0.1) | NAa |

a Values in parentheses are rate values relative to wild type.

A1 subunit cleavage rates of the P4-P3′ factor VIIIa variants. A1 subunit and A1336 product are visualized by Western blotting of factor VIIIa variants. Table 3 shows cleavage rate values for the factor VIII WT and variants. Results for rates of Arg336 cleavage paralleled those for inactivation and are similar in value. Replacing the native P4-P3′ sequence flanking Arg336 with the P4-P3′ flanking Arg740 and Arg372 resulted in 5.6- and 1.7-fold increases in cleavage rates at Arg336, respectively. These results were consistent with the flanking sequences influencing the temporal nature of cleavage events, with activating cleavages preceding the inactivating cleavage and with cleavage at Arg740 preceding that at Arg372. Interestingly, disparate effects were observed with replacement of either prime or nonprime sequences. We observed that the variant 336(P4-P2)372 was cleaved at ~20% the rate of WT, whereas the 336(P1′-P3′)372 variant was cleaved at ~4-fold the rate of WT. Thus the effect caused by the prime side residues appeared to dominate the overall cleavage event in the P4-P3′ substitution, suggesting the marked preference for residues 373SVA379 over 537MKN539. On the other hand, both prime and nonprime sequences flanking Arg740 yielded increased rates of cleavage of 2.9- and 1.5-fold, respec-

(continued)

(Fig. 2). Densitometry scans were used to quantify amounts of intact A1 subunit and the cleaved A1336 subunit over the reaction time course. Progress curves were fitted by nonlinear least squares regression analysis over the initial time points, and rate values were determined as described under “Experimental Procedures.” Table 3 shows cleavage rate values for the factor VIII WT and variants. Results for rates of Arg336 cleavage paralleled those for inactivation and are similar in value. Replacing the native P4-P3′ sequence flanking Arg336 with the P4-P3′ flanking Arg740 and Arg372 resulted in 5.6- and 1.7-fold increases in cleavage rates at Arg336, respectively. These results were consistent with the flanking sequences influencing the temporal nature of cleavage events, with activating cleavages preceding the inactivating cleavage and with cleavage at Arg740 preceding that at Arg372. Interestingly, disparate effects were observed with replacement of either prime or nonprime sequences. We observed that the variant 336(P4-P2)372 was cleaved at ~20% the rate of WT, whereas the 336(P1′-P3′)372 variant was cleaved at ~4-fold the rate of WT. Thus the effect caused by the prime side residues appeared to dominate the overall cleavage event in the P4-P3′ substitution, suggesting the marked preference for residues 373SVA379 over 537MKN539. On the other hand, both prime and nonprime sequences flanking Arg740 yielded increased rates of cleavage of 2.9- and 1.5-fold, respec-
tively, over WT suggesting an overall additive effect on rate consistent with the value as observed for the full P4-P3’ swap of this sequence. Whereas the R336Q variant was refractory to cleavage at Arg336, the 336(P4-P3’)562 variant showed a cleavage rate that was \( \sim 40\% \) the WT value consistent with this sequence representing a less reactive site.

Factor Xa-catalyzed Activation and Inactivation of P4-P3’ Factor VIII Variants—The above results show marked alterations in rates of cleavage at Arg336 dependent upon sequences flanking the scissile bond. These altered rates, such as acceleration in the rate of attack at Arg336, may impact the capacity for factor Xa to activate factor VIII as a result of competing inactivating cleavage. To test this hypothesis, a series of experiments were performed where factor VIII variants were activated by factor Xa and changes in activity were monitored over a time course. Reactions were run with factor VIII (150 nM) and factor Xa (10 nM) in the presence of phospholipid (100 \( \mu \)g/ml), aliquots were removed at the indicated times, and activity was determined by a one-stage clotting assay (Fig. 3). Results using WT factor VIII showed an \( \sim 5\)-fold increase in activity following addition of factor Xa that was followed by a drop in activity to the pre-activated level after about 5 min (Fig. 3A). Interestingly, factor VIII variants that exhibited the fastest rates of cleavage at Arg336 showed reduced peak activities relative to wild type. For example, the 336(P4-P3’)740 variant showed an \( \sim 2\)-fold increase in activity that was short-lived and decayed to its preactivated level by \( \sim 3\) min. Conversely, the factor VIII variants exhibiting rate reductions for cleavage at Arg336 yielded peak activity somewhat greater than WT and showed slower rates of activity decay. For example, the 336(P4-P2)732 variant was activated \( \sim 7\)-fold and still retained \( >5\)-fold the preactivated activity level at 10 min. A similar effect was observed for the R336Q variant. These results suggest that the inability to (efficiently) cleave the scissile bond at Arg336 results in enhanced levels of procofactor activation and greater persistence of factor VIIIa activity.

Western blotting of select factor VIII variants was employed in reactions similar to those above to assess the generation of both the A1 subunit resulting from factor VIII activation and the cleaved A1336 subunit derived from inactivation. Fig. 3B shows results obtained for selected variants from densitometry scans of the blots used to quantitate A1 and A1336 subunits as a percentage of the total density of all antibody-reactive factor VIII bands. The top panel of Fig. 3B shows results obtained for WT factor VIII. Here we observed rapid appearance of the A1 subunit, and a lag in the generation of the A1336 subunit, with the appearance of the latter paralleling the loss of the former.

**FIGURE 3.** Changes in activity of P4-P3’ factor VIII mutant following reaction with factor Xa. Factor VIII (150 nM) was reacted with factor Xa (10 nM) in the presence of phospholipid (100 \( \mu \)g/ml) as described under “Experimental Procedures.” A, changes in activity were measured and plotted for WT (○), 336(P4-P2)372 (●), 336(P1-P3’)372 (○), 336(P4-P3’)372 (□), 336(P4-P2)740 (●), 336(P1-P3’)740 (●), 336(P4-P3’)740 (×), 336(P4-P3’)562 (□), and R336Q (Δ) by one-stage clotting assays. Experiments were performed at least three times with mean \( ±\) S.D. within 20%. B, single chain, heavy chain, A1 subunit, and A1336 products were visualized by Western blotting of factor VIII proteins using the 58.12 antibody following reaction with factor Xa. Densitometry was used to measure appearance and loss of A1 subunit (solid lines) and appearance of the A1336 cleavage product (dashed lines). Concentrations of A1 and A1336, determined by band density of the indicated subunit divided by total densities of all factor VIII reactive bands and multiplied by the initial concentration of factor VIII, were plotted as a function of time for WT (top, ●), 336(P4-P2)372 (middle, ■), and 336(P4-P3’)740 (bottom, ×).
The middle panel shows results for the 336(P4-P2)372 variant, which yielded enhanced levels of activation. This variant showed moderately increased levels of A1 subunit relative to WT, which persisted throughout the time course with little A1336 fragment being generated. On the other hand, results in the bottom panel show the 336(P4-P3)’740 variant in sharp contrast to those in middle panel. This variant demonstrated near equivalent amounts of A1 and A1336 subunits at the initial time point, indicating that inactivation of factor VIII occurred before a significant fraction of these molecules could be activated. As this reaction proceeded, the A1 subunit quickly diminished, whereas the A1336 subunit increased to near maximal levels at ~10 min. Taken together with the above activity results, these data indicate that modulating rates of the inactivating cleavage at Arg336 alters the capacity for factor Xa to activate factor VIII.

Thrombin Cleavage of Arg336 for FVIII Variants—Although thrombin does not cleave at Arg336 in WT factor VIII at an appreciable rate, the substitution of selected residues, especially those flanking the Arg740 site, a site rapidly cleaved by thrombin, could potentially increase the reactivity of thrombin toward this scissile bond. We note that in the WT sequence, residues P3, P2, P1’, and P2’ flanking Arg336 are nonoptimal for thrombin, whereas these residues flanking Arg740 represent optimal residues for thrombin cleavage (26). To assess whether selected variants were cleaved by thrombin at Arg336, we used Western blotting of reactions where variant factor VIII was reacted with thrombin over a 30-min time course. Results are shown for WT and the 336(P4-P3)’740 variant (Fig. 4). In both cases, we observed significant amounts of A1 subunit at 1 min with little if any A1336 product formed over the reaction time course. This observation is consistent with the apparent lack of effect of the mutations on the capacity for thrombin to activate all factor VIII variants by >15-fold (data not shown).

Thrombin Generation Assay for P4-P3’ FVIII Variants—The variants examined in this report exhibited activity levels in one-stage assays that suggested a normal phenotype. However, the marked alterations in rates of factor VIIIa inactivation resulting from cleavage at the Arg336 site could potentially impact stability of factor Xase over time. To assess these effects, thrombin generation assays were examined over an extended time course. In these experiments, hemophilic plasma was supplemented with each variant factor VIII (1 nM) and reactions were initiated with tissue factor (0.15 pM) as described under “Experimental Procedures.” Fig. 5 illustrates results obtained with selected variants with parameter values for all variants presented in Table 4. We observed that eliminating or reducing the rate of cleavage at Arg336 yielded significant increases in parameter values. For example, the R336Q variant showed 2.6- and 1.6-fold increases in thrombin peak value and endogenous thrombin potential, respectively, compared with WT, whereas these values for the 336(P4-P3)’562 variant, which showed ~40% the cleavage rate at this site as compared with WT, were increased ~1.3-fold. On the other hand, variants where cleavage rates at Arg336 were increased, such as the 336(P4-P3)’372 and 336(P4-P3)’740 variants, showed peak values and endogenous thrombin potential that were 10–20% the WT value. Thus specific sequences that flank Arg336 exhibit a significant impact on the generation of thrombin in plasma. Overall, these results indicate that cleavage of factor VIII and VIIIa at Arg336 by factor Xa is modulated by sequences flanking that scissile bond, and that replacing the authentic sequence with those sequences flanking other scissile bonds that are native to the protein and associated with procofactor activation, yield significant acceleration in the rates of inactivation. Furthermore, these data suggest that the product of factor Xase, factor Xa, may be self-regulating as a result from its capacity to feed-back inactivate the cofactor factor VIIIa.

DISCUSSION

Factor Xa exhibits a dual activity toward factor VIII. Like thrombin, factor Xa activates the procofactor by catalyzing cleavages at Arg772, Arg774, and Arg1686; with cleavage at Arg740...
occurring rapidly, whereas cleavage at Arg372 appears rate-limiting. Furthermore, like APC, factor Xa inactivates factor VIII (and factor VIIIa) catalyzing cleavages at Arg336 and Arg562, with attack at the former site correlating with loss of function. In this report we show that sequences flanking these scissile bonds contribute in a primary way to the rate of proteolytic cleavage leading to loss of cofactor function following factor Xa-catalyzed cleavage at Arg336. Specifically, the replacement of P4-P3’ sequences flanking Arg336 with those from the faster-reacting bonds flanking Arg336 and Arg372 increase both rates of cleavage and factor VIII inactivation, whereas replacement of this site with the slow-reacting Arg562 flanking sequence reduces rates of cleavage and consequent inactivation catalyzed by factor Xa. Thus, these results suggest a model whereby factor Xa-catalyzed activation and inactivation of factor VIII (factor VIIIa) are competing processes with relative reaction rates defined in part by the composition of residues directly flanking the scissile bonds.

Little is known about the physiologic regulation of factor VIII activity provided by factor Xa. Factor VIII activation is thought to proceed largely through action by thrombin (see Ref. 3 for review), although a role for factor Xa in this pathway cannot be excluded. Factor VIIIa inactivation leading to denaturing of factor Xase may occur via catalysis by APC and/or factor Xa, as well as the spontaneous decay of factor VIIIa attributed to A2 subunit dissociation. Computational modeling of coagulation reactions indicate a contribution of this nonproteolytic pathway to the decay of factor Xase activity (27). However, it is intriguing to speculate a role for factor Xa, the product of factor Xase, in down-regulating this enzyme complex. Plasma-based thrombin generation assays presented in this report showed improved parameter values including peak thrombin and endogenous thrombin potential as compared with WT factor VIII when cleavage at Arg336 was slowed by selective replacement of flanking P4-P3’ residues or when cleavage was prevented in the R336Q mutation. Inasmuch as the thrombin generation assay is a cell-free assay with no thrombomodulin present, the contributions of APC to impacting factor VIIIa activity are likely minimal if any at all. Thus these results strongly suggest that factor Xase is down-regulated in part by its product, factor Xa.

All factor VIII flanking sequence variants examined in this report possessed specific activity values indicative of a normal phenotype. Although some differences in these values were observed, they did not appear to correlate with enhanced or reduced rates of cleavage at Arg336. This result was consistent with the one-stage assay used to monitor this activity assessing the initial generation of thrombin rather than total thrombin. Furthermore, whereas thrombin efficiently activated all factor VIII variants employed in this study yielding peak activity values >15-fold above the basal level, factor Xa-catalyzed activation of the factor VIII variants was variable. Increasing the rates of cleavage at Arg336 in factor VIII following replacement of this flanking sequence with that of Arg562 yielded marked reductions in the peak factor VIIIa activity. This result correlated with the enhanced proteolytic inactivation of the procotfactor limiting the available functional factor VIII that could be activated. Thrombin generation assays using these “enhanced cleavage” variants showed marked reductions in peak thrombin and endogenous thrombin potential parameters. These results derive in part from enhanced down-regulation of factor Xase by increased proteolysis catalyzed by factor Xa at the Arg336 site. Furthermore, limited activation of these variants by factor Xa could also potentially limit available factor VIIIa, which in turn would limit thrombin generation. Indeed, the effects observed on thrombin generation may derive from a combination of factor Xase inhibition and reduced factor VIIIa generation as a result of factor Xase activity. At present, we are not able to discriminate a dominant mechanism for this effect.

The catalysis of factor VIII (factor VIIIa) by factor Xa is driven by exosite-dependent interactions. Interactive sites for factor Xa have been mapped to residues 2253–2270 in the C2 domain (28) and residues 337–372 in the a1 segment at the C-terminal end of A1 subunit (29). An acidic segment within the catalytic site corresponding to Asp361–Asp362–Asp363 appears to make important contributions to this interaction (29), which also contributes to a binding site for zymogen FX binding (30). Solid phase binding assays indicated that the affinity of factor Xa for the isolated A1 subunit of factor VIIIa is ~20-fold greater than the affinity of factor X for this subunit suggesting that binding of the zymogen may offer little protection from attack by factor Xa.

As with other coagulation serine proteases, exosite-dependent interactions likely modulate the $K_m$ parameter value for catalysis by factor Xa (31), with other factors contributing to active site docking and catalytic rate. In an earlier study examining APC-catalyzed cleavage and inactivation of factor VIIIa, which like factor Xa, attacks Arg336 initially and Arg562 second-
Modulation of Factor Xa Cleavage by Flanking Sequences

arly, we showed that swapping the P4-P3’ sequence flanking Arg336 with that from Arg262 resulted in a near 100-fold reduction in the rate of cleavage at Arg336 (15). This rate reduction was attributed to replacement of critical flanking residues with residues less optimal for cleavage. Similar to that study, the rate differences observed for factor Xa catalysis of the Arg336 flanking sequence variants appeared to reflect the presence or absence of cleavage optimal residues. Of the variants tested, the greatest increase (~6-fold) in rate of Arg336 cleavage, and consequent rate of factor VIIIa inactivation was observed for the 336(P4-P3’)740 variant. This rate increase likely derived from more optimal cleavage residues present in both the nonprime and prime sides of the scissile bond as indicated by rate increases for the 336(P4-P2)740 and 336(P1’-P3’)740 variants of 1.5- and 2.9-fold, respectively, relative to WT. Interestingly, the modest overall increase in cleavage rate observed for the 336(P4-P3’)372 variant (1.7-fold over WT) appeared to result from the combination of a rate reduction observed for the 336(P4-P2)372 variant (~20% the WT value) that was compensated for by an ~4-fold rate increase for the 336(P1’-P3’)372 variant.

These observations correlated well with results from Bianchini et al. (32) who identified the contributions of individual residues for factor Xa cleavage by examining catalytic efficiency using a series of fluorescence-quenched peptide substrates. Results from that study mapping each amino acid residue at P3-P3’ positions in the peptide substrates revealed maximal selectivity differences in the P2 and P1’ positions. Thus the faster cleavage of both the Arg372 and Arg404 P1’-P3’ variants (both contain Ser at the P1’ position) over the WT was likely attributed to the ~10-fold increase in catalytic efficiency for peptide cleavage with a P1’ Ser residue compared with a P1’ Met residue. Furthermore, the reduced rate observed with the 336(P4-P2)372 variant could reflect the ~3-fold decrease in catalytic efficiency for a P2 Ile residue (found in the 372 sequence) compared with the P2 Leu residue (present in the native sequence). On the other hand, the modest increase in rate for the 336(P4-P2)740 variant (P2 Pro) over WT (P2 Leu) may reflect the ~2-fold increase in catalytic efficiency for a peptide containing a P2 Pro compared with Leu at this position. Examination of the P3-P3’ sequence flanking the Arg462 site suggests the slower rate of cleavage for the 336(P4-P3’)562 variant (~40% compared with native factor VIII) may have derived from differential contributions from several residues, the most prominent represented by an Asp at the P3 position, which yielded an ~5-fold decrease in catalytic efficiency compared with P3 Gln in the peptide substrate.

In conclusion, the results from this study indicate that sequences flanking scissile bonds in factor VIII attacked by factor Xa contribute to proteolytic rates and influence pathways leading to both the factor VIII activation and inactivation catalyzed by this enzyme. These results also support a physiologic role for factor Xa-catalyzed inactivation of factor VIIIa with consequent dampening of factor Xase as judged by decreases in thrombin generation parameter values when this cleavage rate is increased and enhanced parameter values with reduction or elimination of this cleavage.

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