Acidic Residues C-Terminal to the A2 Domain Facilitate Thrombin-Catalyzed Activation of Factor VIII[†]

Jennifer L. Newell and Philip J. Fay*

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, New York 14642

Received May 1, 2008; Revised Manuscript Received June 18, 2008

ABSTRACT: Factor VIII is activated by thrombin through proteolysis at Arg740, Arg372, and Arg1689. One region implicated in this exosite-dependent interaction is the factor VIII a2 segment (residues 711−740) separating the A2 and B domains. Residues 717−725 (DYYEDSYED) within this region consist of five acidic residues and three sulfo-Tyr residues, thus representing a high density of negative charge potential. The contributions of these residues to thrombin-catalyzed activation of factor VIII were assessed following mutagenesis of acidic residues to Ala or Tyr residues to Phe and expression and purification of the B-domainless proteins from stable-expressing cell lines. All mutations showed reduced specific activity from ∼30% to ∼70% of the wild-type value. While replacement of the Tyr residues showed little, if any, effect on rates of thrombin-catalyzed proteolysis of factor VIII and consequent activation, the acidic to Ala mutations Glu720Ala, Asp721Ala, Glu724Ala, and Asp725Ala showed decreased rates of proteolysis at each of the three P1 residues. Mutations at residues Glu724 and Asp725 were most affected with double mutations at these sites showing ∼10-fold and ∼30-fold reduced rates of cleavage at Arg372 and Arg1689, respectively. Factor VIII activation profiles paralleled the results assessing rates of proteolysis. Kinetic analyses revealed these mutations minimally affected apparent V_{max} for thrombin-catalyzed cleavage but variably increased the K_m for procofactor up to 7-fold, suggesting the latter parameter was dominant in reducing catalytic efficiency. These results suggest that residues Glu720, Asp721, Glu724, and Asp725 likely constitute an exosite-interactive region in factor VIII facilitating cleavages for procofactor activation.

Factor VIII is essential in blood coagulation as seen by defects or deficiencies in the glycoprotein resulting in the severe inherited bleeding disorder, hemophilia A. Factor VIII is synthesized as an ∼300 kDa single chain protein containing 2332 amino acids and has six domains that are structurally designated based on internal sequence homology as NH_2-A1-A2-B-A3-C1-C2-COOH (*1, 2*). Additionally, there are short regions following the A1 and A2 domains and preceding the A3 domain, designated a1 (residues 337−372), a2 (residues 711−740), and a3 (1649−1689), that contain high concentrations of acidic residues. Factor VIII circulates as a series of heterodimers formed by cleavage at the B-A3 junction and by several cleavages within the B domain. Thus, circulating factor VIII is a divalent metal ion-dependent heterodimeric protein comprised of a heavy chain (A1-a1-A2-a2-B domains) and a light chain (a3-A3-C1-C2 domains) (*3*).

The activated form of factor VIII, factor VIIIa, functions as a cofactor for factor IXa, increasing its catalytic efficiency by several orders of magnitude in the factor Xase-catalyzed conversion of factor X to factor Xa (*4*). The inactive factor VIII procofactor is converted to factor VIIIa through limited proteolysis catalyzed by either thrombin or factor Xa (*5, 6*), with thrombin being the more catalytically efficient activator of factor VIII (*5–7*). Activation of factor VIII occurs through proteolysis by either protease via cleavage at the P1 residues Arg740 (A2-B domainal junction), Arg372 (A1-A2 domainal junction), and Arg1689 (a3-A3 junction) (*5*). Following procofactor activation, a weak electrostatic interaction exists between the A1/A3-C1-C2 dimer and A2 subunit (*8, 9*), and dissociation of the A2 subunit inactivates the cofactor and consequently dampens factor Xase (*3*).

Thrombin cleavage of factor VIII appears to be an ordered pathway, with initial proteolysis at Arg740 facilitating subsequent proteolysis at Arg372 and Arg1689 (*10*). Cleavage at Arg372 exposes a cryptic functional factor IXa-interactive site in the A2 domain (*11*), while cleavage at Arg1689 liberates factor VIII from von Willebrand factor (*12*) and contributes to factor VIIIa specific activity (*13, 14*), thus making both sites essential for procofactor activation.

Anion-binding exosites figure prominently in the mechanism for thrombin-catalyzed activation of factor VIII (*15–17*). However, little information is available on thrombin-interactive sites in the factor VIII substrate. Results from an earlier report suggested that the C-terminal region of the A2 subunit (a2 region) contributed to thrombin binding and facilitated proteolysis (*17*). In this report we examine a sequence of high negative charge density (residues 717−725) in the a2 region that appears to constitute an exosite-interactive site.

[†] This work was supported by Grants HL38199 and HL76213 from the National Institutes of Health. J.L.N. is supported by NIH Predoctoral Training Grant T32-HL07512. An account of this work was presented at the 49th meeting of the American Society of Hematology, Atlanta, GA, Dec 9, 2007.

* To whom correspondence should be addressed. Tel: 585-275-6576. Fax: 585-275-6007. E-mail: philip_fay @urmc.rochester.edu.

10.1021/bi8007824 CCC: $40.75
Published on Web 07/22/2008

This segment contains five acidic residues as well as three sulfo-Tyr residues. Results from mutagenesis studies where acidic residues are individually replaced with Ala and Tyr residues replaced with Phe suggest a contribution from selective acidic residues but not the Tyr residues in facilitating thrombin-catalyzed proteolysis at each of the P1 Arg residues.

MATERIALS AND METHODS

Reagents. The monoclonal antibodies C5 (recognizing the a1 region) and 2D2 (recognizing the A3 domain) were generous gifts from Zaverio Ruggeri and Bayer, respectively. The anti-A2 domain factor VIII monoclonal antibody R8B12 (*8*) was obtained from Green Mountain Antibodies. The monoclonal antibody ESH8 (*18*) recognizing the C2 domain was purchased from American Diagnostica. The reagents human α-thrombin, factor IXa, factor X, and factor Xa were purchased from Enzyme Research Laboratories. Recombinant nonsulfated hirudin and horseradish peroxidase-labeled streptavidin were from Calbiochem. Chromogenic factor Xa substrate Pefa-5523 (Pefachrome FXa) was purchased from Centerchem. Factor VIII-deficient plasma was prepared as previously described (*19*). Phospholipid vesicles containing 20% phosphatidylserine, 40% phospatidylcholine, and 40% phospatidylethanolamine were prepared using *n*-octyl glucoside as previously described (*20*). The Bluescript factor VIII vector (pBS factor VIII) and B-domainless factor VIII expression construct RENeo factor VIII were kindly provided by Pete Lollar and John Healey (Emory University).

Construction, Expression, and Purification of Recombinant Factor VIII. B-domainless factor VIII cDNA was restricted from the factor VIII expression construct FVIIIHSQ-MSAB-NotI-RENeo using the endonucleases *Xho*I and *Not*I and cloned into the pBluescript II K/S-vector. The B-domainless factor VIII cDNA was further restricted using endonucleases *Sac*II/*Apa*I and subcloned into the pBluescript II K/S-vector. The factor VIII mutants Asp717Ala, Tyr718Phe, Tyr719Phe, Glu720Ala, Asp721Ala, Tyr723Phe, Glu724Ala, Asp725Ala, Tyr718Phe/ Tyr719Phe/Tyr723Phe, Glu720Ala/Asp721Ala, and Glu724Ala/Asp725Ala were introduced into the construct using the Stratagene QuickChange site-directed mutagenesis kit as previously described (*21*). The presence of only the desired mutation was confirmed using dideoxy sequencing. The mutated factor VIII cDNA was then ligated back into the factor VIII expression construct and subjected to a second round of dideoxy sequencing to confirm that only the desired mutation was present. FuGENE6 (Roche) was used to transfect the factor VIII expression vector into BHK[1] cells. The selection, subcloning, and cloning of stable transfectants were performed by standard methods, and the cloned cells were cultured in roller bottles for protein expression (*22*). The conditioned media were collected daily, and the expressed proteins were purified by SP-Sepharose (Amersham Biosciences) column chromatography as previously described (*22*). A one-stage clotting assay was used to detect active fractions. Yields of purified wild-type and variant factor VIII ranged from 0.1 to 0.4 mg/L of conditioned media. Resultant factor VIII was >90% pure as judged by SDS−PAGE with the main contaminant being albumin. Factor VIII samples were quick-frozen and stored at −80°C.

ELISA. The concentration of purified factor VIII proteins was determined by a sandwich ELISA. ESH8 was used as the capture antibody, and biotinylated R8B12 was used as the detection antibody. The amount of factor VIII on the plate was determined as previously described (*21*) using a chromogenic assay utilizing streptavidin-linked horseradish peroxidase (Calbiochem) with the chromogen *o*-phenylenediamine dihydrochloride (Sigma). Purified commercial recombinant factor VIII (Kogenate; Bayer Corp.) was used as the standard. Factor VIII specific activity values were determined using one-stage clotting and ELISA (*21*).

Factor Xa Generation Assay. The rate of conversion of factor X to factor Xa was monitored in a purified system (*23*). For the factor VIII activation time course following thrombin addition, factor VIII (1 nM) was reacted with thrombin (0.05 nM) in the presence of phospholipid vesicles (10 μM) at 22 °C. Samples were removed at indicated times, and thrombin activity was inhibited by the addition of hirudin (0.1 unit/mL). Factor Xa generation was initiated by addition of factor IXa (20 nM) and factor X (300 nM). The reactions were terminated with EDTA (50 mM) at the indicated times. Rates of factor Xa generation were determined by the addition of the chromogenic substrate Pefa-5523 (0.46 mM final concentration). Reactions were read at 405 nm for 5 min using a V_{max} microtiter plate reader (Molecular Devices). To assess the K_m(app), various concentrations of wild-type and mutant factor VIII were reacted with thrombin (0.05 nM) for 15 s. Thrombin was inactivated by addition of hirudin (0.1 unit/mL) in the presence of phospholipid vesicles (10 μM), and each sample was reacted with factor IXa (20 nM) and factor X (300 nM). Aliquots were removed at appropriate times to assess initial rates of product formation, added to tubes containing EDTA (50 mM final concentration), and processed as described above.

Cleavage of Factor VIII by Thrombin. Factor VIII (100 nM) was reacted with 2.5 nM thrombin in a buffer containing 20 mM HEPES (pH 7.2), 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20. Reactions were run at 22 °C, and samples were taken at indicated times. The reactions were terminated by addition of SDS−PAGE sample buffer and boiling for 3 min.

Electrophoresis and Western Blotting. Samples were run by SDS−PAGE on 8% polyacrylamide gels. Electrophoresis was carried out using a Bio-Rad mini gel apparatus at 175 V for 1 h. Proteins were transferred to polyvinylidene fluoride membrane for Western blotting. Blots were probed using the anti-factor VIII monoclonal antibodies indicated in the figure legends, followed by reaction with a goat anti-mouse alkaline phosphatase-linked secondary antibody (Sigma). The signal was detected using the ECF (enhanced chemifluorescence) system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices). Densitometric scans were quantitated from linear density regions of the blots using Image Quant software (Molecular Devices).

Data Analysis. All experiments were performed at least three separate times, and the average values are shown. Western blots were analyzed by densitometry and nonlinear least squares regression, and the initial time points were fitted to the single exponential equation:

[1] Abbreviations: BHK, baby hamster kidney; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; WT, wild-type; CHO, Chinese hamster ovary.

$$A = A_o(1 - e^{-kt}) \quad (1)$$

where A_o is the total A1, A2, or A3-C1-C2 generated in nanomolar, k is the rate constant in min^{-1}, and t is the time in minutes.

Kinetic parameters were determined by Xa generation analysis. K_m and V_{max} were calculated from initial rate data by fitting the data using nonlinear least-squares regression analysis to the Michaelis−Menton equation:

$$v_o = (V_{max}[S])/(K_m + [S]) \quad (2)$$

where v_o is the initial velocity in nanomolar per minute and [S] is the concentration of wild-type or mutant factor VIII in nanomolar. Using these conditions, the V_{max} recorded is for factor Xa generation and relates directly to the amount of factor VIIIa formed, while K_m is a measure of the concentration of substrate factor VIII acted upon by thrombin.

RESULTS

Characterization of Recombinant Factor VIII Proteins. Factor VIII residues 717−725 (DYYEDSYED) represent a region of high negative charge density localized to the a2 segment separating the A2 and B domains of factor VIII. In addition to the acidic residues, this sequence also contains three Tyr residues at positions 718, 719, and 723 that were shown to be modified by posttranslational sulfation (*24, 25*). Thus, this modification also contributes a negative charge potential that could influence the anion-binding exosite-dependent activation of factor VIII by thrombin.

In order to determine whether these residues contributed to thrombin-catalyzed activation of factor VIII, we prepared a number of point mutations where each of the acidic residues in the 717−725 segment was replaced with Ala. In addition, two double mutations were prepared where Glu720/Asp721 and Glu724/Asp725 were replaced with Ala. We also prepared a set of reagents where individual Tyr residues were replaced with Phe to prevent O-sulfation at that site as well as the triple mutant Tyr718Phe/Tyr719Phe/Tyr723Phe. All variants were stably expressed as B-domainless factor VIII in BHK cells, and protein was purified as described in Materials and Methods. Specific activity values for wild type and mutants are shown in Table 1. The specific activities for the acidic to Ala variants were ~70% the wild-type value for Asp717Ala and ~50% for Glu720Ala, Asp721Ala, Glu724Ala, and Asp725Ala. The double mutants of Glu720Ala/Asp721Ala and Glu724Ala725Ala had slightly lower specific activity than the individual point mutants. Similarly, the Tyr to Phe mutations also showed ~30% to ~70% the wild-type value with the triple mutant possessing the lowest overall activity. The activity of the triple mutant was similar to that reported in an earlier study (~50% the wild-type value (*26*)). Together, these results suggest the point mutations retained somewhat variable but reduced specific activity values as compared to wild-type factor VIII and would generally represent near-normal phenotypes.

Thrombin-Catalyzed Cleavage of Acidic Residue to Ala Factor VIII Variants and the Generation of A1 Subunit. To examine the effect of the acidic residues on activating cleavages in factor VIII, reactions were initiated using catalytic levels of thrombin (2.5 nM) relative to factor VIII (100 nM) and run over a 25 min time course. Results were visualized using SDS−PAGE and Western blotting. Wild-type or mutant B-domainless factor VIII expressed in BHK cells is secreted in two forms, a single chain (contiguous heavy chain and light chain) and the heterodimer, in near equivalent amounts. Results from time courses of the thrombin-catalyzed cleavage reactions of wild-type and mutant proteins probed using the anti-A1 domain specific monoclonal antibody, C5, are shown in Figure 1. Reaction of wild-type factor VIII with thrombin showed efficient cleavage of both single chain and heavy chain resulting in the rapid generation of the A1 subunit.

Table 1: Specific Activity of Wild-Type and Mutant Factor VIII[a]

factor VIII	specific activity (%)	factor VIII	specific activity (%)
wild type	100 ± 5	Y723F	73 ± 7
D717A	72 ± 3	E724A	56 ± 4
Y718F	31 ± 3	E725A	46 ± 2
Y719F	45 ± 4	Y718/19/23F	27 ± 3
E720A	44 ± 2	E720/1A	33 ± 4
D721A	57 ± 2	E724/5A	32 ± 4

[a] Specific activity values were determined as described under Materials and Methods and are presented as a percentage ± standard deviation of the wild-type value.

When examining thrombin cleavage of the factor VIII mutants, results revealed overall reduced rates of cleavage at Arg372 as seen by retention of factor VIII heavy chain (contiguous A1-A2) with a reduction in A1 subunit generation as compared to wild-type protein (Figure 1). Since the A1 subunit is derived following a single cleavage at Arg372, its generation reflects the rate of cleavage at that site. Results from the blots were quantitated by densitometry and subjected to nonlinear least squares regression analysis (Figure 2) to calculate rates of A1 subunit generation (Table 2). The Asp717Ala and Glu720Ala mutations showed no significant effects on the rate of A1 subunit generation. However, the other single point mutants showed ~2−3-fold reduction in the rate of generation of A1 subunit indicating impaired cleavage at Arg372 compared to wild-type factor VIII (Figure 2, Table 2). These reaction rates were further reduced in the two double mutants where the overall rate for A1 subunit generation was reduced ~4−5-fold compared with wild type. Thus replacement of residues Asp721, Glu724, and Asp725 appeared to affect rates of thrombin-catalyzed cleavage at Arg372 generating the A1 subunit.

Generation of A2 Subunit by Thrombin Cleavage of Factor VIII Variants. A similar approach was used to assess rates of A2 subunit generation using the anti-A2 domain specific antibody R8B12. A2 subunit is derived from a cleavage at Arg372 and Arg740, the latter at the a2-B domain junction. In the B-domainless factor VIII heterodimer, a remnant (~14 residues) of the B domain remains C-terminal to the a2 segment, and its presence or absence cannot be discriminated by SDS−PAGE. Thus the band we visualize as A2 subunit requires only the single cleavage at Arg372 to be released from the heavy chain of the heterodimer, whereas cleavages at both Arg372 and Arg740 are necessary to excise this subunit from single chain factor VIII. For these reasons, results presented for rates of A2 subunit generation give limited information on actual cleavage rates at Arg740 but are useful in assessing rates of A2 subunit generation in the variants relative to the wild type.

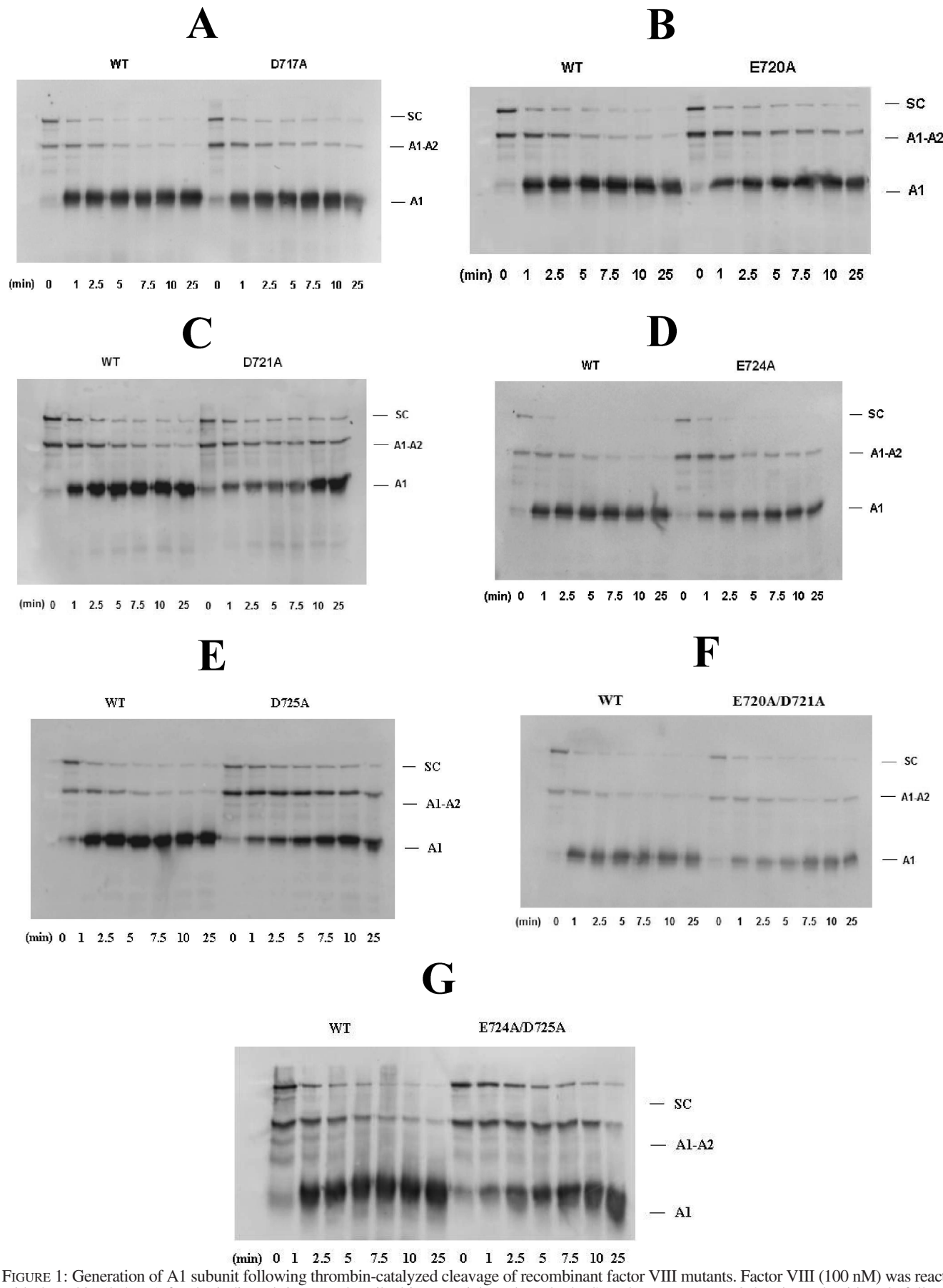

FIGURE 1: Generation of A1 subunit following thrombin-catalyzed cleavage of recombinant factor VIII mutants. Factor VIII (100 nM) was reacted with thrombin (2.5 nM) for the indicated times as described in Materials and Methods. Samples were run on 8% polyacrylamide gels followed by Western blotting using an anti-A1 antibody. Blotting data are as follows: (A) Asp717Ala, (B) Glu720Ala, (C) Asp721Ala, (D) Glu724Ala, (E) Asp725Ala, (F) Glu720Ala/Asp721Ala, and (G) Glu724Ala/Asp725Ala. The abbreviations WT, A1-A2, and SC represent wild type, heavy chain (contiguous A1-A2 domains), and single chain, respectively.

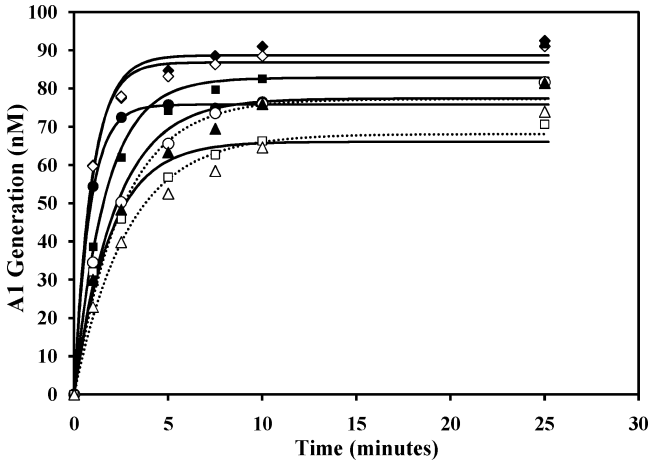

FIGURE 2: A1 subunit generated following thrombin cleavage of factor VIII. Wild-type and mutant recombinant factor VIII (100 nM) were reacted with thrombin (2.5 nM) for the indicated times as described in Materials and Methods. Samples were run on 8% polyacrylamide gels followed by Western blotting using an anti-A1 antibody. Data was derived from densitometric analysis of A1 generation (nM) from blotting data (Figure 1). Symbols: ◆, wild type; ◇, Asp717Ala; ●, Glu720Ala; ○, Asp721Ala; ■, Glu724Ala; □, Asp725Ala; ▲, Glu720Ala/Asp721Ala; Δ, Glu724Ala/Asp725Ala. The data were fit to the single exponential equation using nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown. Continuous lines represent the single mutants while the double mutants are represented by the dotted lines.

Results from Western blots are presented as Supporting Information with linear regression analysis of scanned blots shown in Figure 3 and rate values presented in Table 2. The rate of A2 subunit generation for the Asp717Ala variant was similar to wild-type factor VIII and equivalent to the rate of generation of the A1 subunit from these factor VIII forms. This latter result is consistent with cleavage at Arg740 representing the fast step in an ordered mechanism for cleavage of the factor VIII heavy chain (*10*). The other single mutations of Glu720Ala, Asp721Ala, Glu724Ala, and Asp725Ala and the double mutant Glu720Ala/Asp721Ala revealed reductions in the rate of A2 subunit generation from ~2- to 5-fold compared to wild-type factor VIII, while the double mutant Glu724Ala/Asp725Ala was reduced nearly 10-fold (Figure 3 and Table 2). Overall, these results suggest that, with the exception of Asp717, all acidic residues in this cluster contribute to thrombin-catalyzed cleavages within the factor VIII heavy chain with the most C-terminal residues showing a somewhat greater effect.

Cleavage at Arg1689 in Factor VIII Variants by Thrombin. The above observations support a model where these a2 region acidic residues differentially reduce thrombin cleavage at Arg372 and Arg740. To directly assess effects of these acidic residues on cleavage at the a3-A3 junction (Arg1689) in the factor VIII light chain, Western blot reactions similar to those above were run with the A3 domain-specific antibody, 2D2. Blotting data evaluating rates of generation of the A3-C1-C2 subunit (see Supporting Information) for the wild type and mutants were quantitated by scanning densitometry, and results are presented in Figure 4. Since generation of A3-C1-C2 subunit is derived from a single cleavage at Arg1689 in either precursor factor VIII forms, rates for cleavage at this site and subunit generation are equivalent. The cleavage rate for thrombin at Arg1689 was reduced ~3-fold for Asp717Ala as compared with the wild-type protein (Table 2 and Figure 4). The mutants Glu720Ala, Asp721Ala, Glu724Ala, and Asp725Ala showed furthered decreased rates for thrombin cleavage with reductions of ~11-, 10-, 15-, and 17-fold, respectively (Table 2). In examining the double mutations the most significant reduction was ~34-fold for Glu724Ala/Asp725Ala, as compared to ~17-fold for the Glu720Ala/Asp721Ala variant (Table 2). Overall, we observed significantly decreased rates for light chain cleavage that in general paralleled the results observed with cleavage at the thrombin sites in the heavy chain. Taken together with the above results, these acidic residues appear to facilitate cleavage at all thrombin sites in the procofactor.

A series of reactions using wild type and the two double mutants were also run under more physiological conditions (37 °C and 140 mM NaCl) to ensure relevance of the above analyses. Results from SDS−PAGE and Western blotting (data not shown) were quantitated as above, and rate data are presented in Figure 5 and Table 3. In general, we observed modest increases in subunit generation rates that maximally approached 2-fold the value observed for the lower temperature and NaCl condition. However, comparison of the relative rates of subunit generation for the two double mutants with the wild type indicated similar fold reductions for the generation of each factor VIIIa subunit. These results indicate that the reduced temperature and NaCl conditions did not appreciably influence the role of the acidic residues in their contribution to the catalytic mechanism.

Thrombin Cleavage of Sulfotyrosine Factor VIII Mutants. An earlier study showed that replacement of the three sulfo-Tyr residues at positions 718, 719, and 723 resulted in modest, if any, effects on the apparent efficiency of thrombin-catalyzed cleavages (*26*). However, those studies employed transiently transfected cell lines, and thrombin activation experiments were performed using a severalfold molar excess of the proteinase relative to the factor VIII substrate.

To gain insights into the role of these residues in the activation mechanism, the single point and the triple mutation variants were assessed using catalytic thrombin concentrations similar to the evaluation of acidic to Ala mutants described above. Wild-type and mutant factor VIII (100 nM) were reacted with thrombin (2.5 nM), and resultant products were visualized using SDS−PAGE and Western blotting as described above. The anti-A2 antibody (R8B12) and the anti-A3 antibody (2D2) were used to assess rates of A2 and A3-C1-C2 subunit generation, respectively. Results show similar rates of A2 and A3-C1-C2 generation for the mutants as compared to wild-type factor VIII (Figure 6). These data suggest that tyrosine sulfation in the A2 domain had little effect on the thrombin-catalyzed cleavages of factor VIII.

Thrombin Activation of Factor VIII Proteins. The generation of cofactor activity from resulting thrombin-catalyzed activation of the wild-type and variant procofactors was investigated using a factor Xa generation assay as described in Materials and Methods (Figure 7). Activity for the wild-type protein increased rapidly, peaking between 1 and 2 min after which this activity level quickly decayed to approximately 10% peak activity at 30 min. The loss of factor Xase activity over the time course results from inactivation of factor VIIIa by dissociation of A2 subunit from the A1/A3-C1-C2 dimer (*3*). Activation of the single point mutants by thrombin produced peak activity values between 1 and 2

Table 2: Rates of Subunit Generation during Factor VIII Activation by Thrombin[a]

factor VIII	rate of A1 subunit generation [nM A1 min^{-1} (nM IIa)$^{-1}$]	rate of A2 subunit generation [nM A2 min^{-1} (nM IIa)$^{-1}$]	rate of A3-C1-C2 subunit generation [nM A3-C1-C2 min^{-1} (nM IIa)$^{-1}$]
wild type	43.8 ± 3.3	32.7 ± 6.4	229 ± 21
D717A	38.8 ± 2.7	38.4 ± 7.5	68.8 ± 2.4
E720A	36.4 ± 4.3	18.1 ± 3.2	20.2 ± 3.4
D721A	13.8 ± 3.0	20.4 ± 3.1	23.4 ± 4.5
E724A	17.9 ± 0.9	8.1 ± 4.2	15.4 ± 3.6
E725A	13.7 ± 4.2	7.1 ± 4.0	12.4 ± 1.8
E720/721A	12.2 ± 1.5	10.8 ± 0.8	13.4 ± 1.7
E724/725A	9.1 ± 1.6	3.7 ± 0.9	6.8 ± 0.9

[a] Subunit generation rates for A1, A2, and A3-C1-C2 subunits by thrombin cleavage of wild-type and mutant factor VIII were estimated by nonlinear regression analysis of the data shown in Figures 2, 3, and 4. Experiments were performed at least three separate times, and average values are shown.

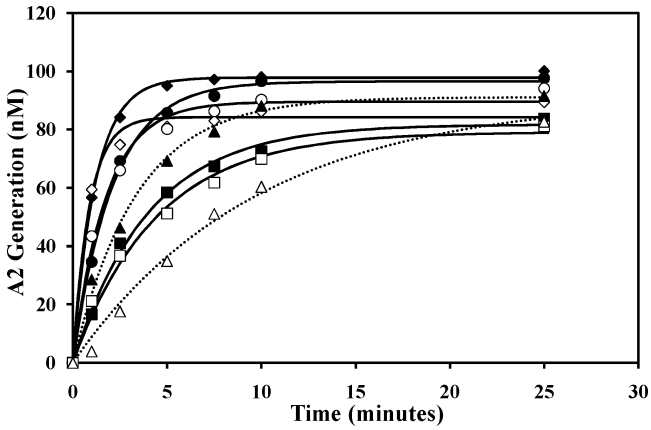

FIGURE 3: Generation of A2 subunit by thrombin-catalyzed proteolysis of recombinant factor VIII. Wild-type and mutant recombinant factor VIII (100 nM) were reacted with thrombin (2.5 nM) for the indicated times as described in Materials and Methods. Samples were run on 8% polyacrylamide gels followed by Western blotting using the anti-A2 antibody R8B12. Data was derived from quantitative densitometry of A2 generation (nM) from blotting data (see Supporting Information for blots). Symbols: ◆, wild type; ◇, Asp717Ala; ●, Glu720Ala; ○, Asp721Ala; ■, Glu724Ala; □, Asp725Ala; ▲, Glu720Ala/Asp721Ala; Δ, Glu724Ala/Asp725Ala. The data were fitted to the single exponential equation using nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown. Continuous lines represent the single mutants while the double mutants are represented by the dotted lines.

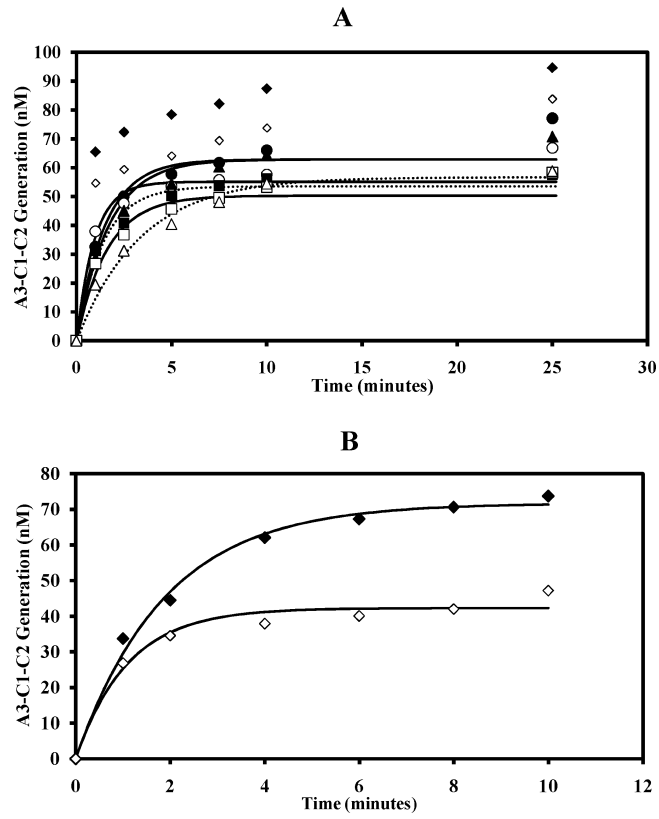

FIGURE 4: Generation of A3-C1-C2 subunit following thrombin cleavage of factor VIII variants. Panel A: Recombinant wild-type and mutant factor VIII (100 nM) were reacted with thrombin (2.5 nM) for the indicated times and subjected to SDS−PAGE and blotting using the 2D2 antibody (see Supporting Information for blots). Densitometry data from blots are plotted as described in the legend to Figure 2. Panel B shows results obtained using wild-type and D717A factor VIII (100 nM) reacted with a lower thrombin concentration (0.125 nM). Symbols: ◆, wild type; ◇, Asp717Ala; ●, Glu720Ala; ○, Asp721Ala; ■, Glu724Ala; □, Asp725Ala; ▲, Glu720Ala/Asp721Ala; Δ, Glu724Ala/Asp725Ala. The data were fitted to the single exponential equation using nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown. Continuous lines represent the single mutants while the double mutants are represented by the dotted lines.

min, similar to the wild-type protein. However, these values were ~2−3-fold lower for the variants than for wild-type factor VIII, and profiles had a broader character. This appearance likely reflects slower rates of activation inasmuch as the typical spike in activity broadens when the factor VIIIa decay rate is unaltered, but procofactor activation rates are reduced (*3*). This interpretation was supported by reacting the double mutant variants with thrombin. Evaluation of Glu720Ala/Asp721Ala and Glu724Ala/Asp725Ala factor VIII forms showed a relatively slow increase in cofactor activity reaching a maximal level at 5−7 min that represented ~30% and 60%, respectively, the level observed for wild type. These data are consistent with a reduced capacity for thrombin to cleave the double mutants as compared to the single mutants. Overall, these results are consistent with Western blot analysis suggesting that these acidic residues are required for efficient generation of factor VIIIa by thrombin.

Kinetic Parameters of Thrombin Activation for Wild-Type and Acidic Residue to Ala Mutants. Since several of the acidic residue to Ala, but not the Tyr to Phe variants, demonstrated altered interactions with thrombin, the kinetics for procofactor activation for these variants were measured using the factor Xa generation system as described in Materials and Methods. Variable amounts of wild type and selected acidic residue to Ala factor VIII variants were activated with a catalytic thrombin concentration (0.05 nM) for 15 s in order to ensure initial rate conditions. Subsequent addition of phospholipid vesicles (10 μM) and a high concentration of factor IXa (20 nM) was used to drive factor

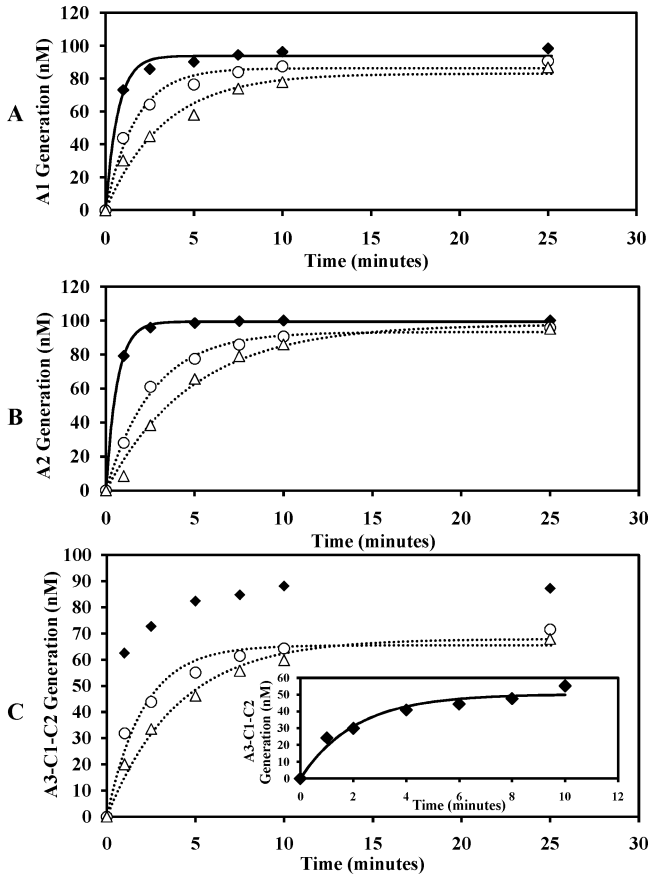

FIGURE 5: Generation of A1, A2, and A3-C1-C2 subunits at 37 °C and 140 mM NaCl following thrombin-catalyzed cleavage of recombinant wild-type and double mutant factor VIII. Wild-type and mutant recombinant factor VIII (100 nM) were reacted with thrombin (2.5 nM) at 37 °C in buffer containing 140 mM NaCl for the indicated times as described in Materials and Methods. Samples were run on 8% polyacrylamide gels and subjected to Western blotting with the following antibodies: (A) C5 (anti-A1), (B) R8B12 (anti-A2), and (C) 2D2 (anti-A3). Data were derived from densitometric analysis of A1, A2, and A3-C1-C2 subunit generation (nM) from blotting data (data not shown). Symbols: ◆, wild type; ○, Glu720Ala/Asp721Ala; Δ, Glu724Ala/Asp725Ala. The insert in panel C shows results obtained using wild-type factor VIII (100 nM) reacted with a lower thrombin concentration (0.125 nM). The data were fit to the single exponential equation using nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown. The continuous line represents wild-type factor VIII while the double mutants are represented by the dotted lines.

VIIIa into formation of the intrinsic factor Xase, and factor Xa generation reactions were initiated by addition of factor X (300 nM). Under these conditions, the resultant rate of product factor Xa formation is directly proportional to the concentration of factor VIIIa in the reaction and can be used to assess the rate of thrombin-catalyzed conversion of procofactor to cofactor. Data from thrombin activation of factor VIII were fitted to the Michaelis−Menten equation using nonlinear least squares regression (Table 4 and Figure 8). V_{max} values for factor Xa generated were similar for the wild-type and variant factor VIII indicating that once activated, the cofactor activities for these proteins were not affected by the mutations (Table 4). K_m(app) values were minimally increased for the Glu720Ala and Asp721Ala variants with the double mutation at these sites showing an ∼2-fold increase in this parameter value compared to wild type (Table 4). However, the Glu724Ala and Asp725Ala variants showed ∼4−5-fold increases in K_m(app) with the double mutation showing an ∼7-fold increase (Table 4). These data were consistent with the acidic residues contained within the a2 segment making varied contribution to thrombin binding the factor VIII substrate such that loss of charge in this region reduced affinity for this interaction, impacting catalytic efficiency for procofactor activation.

DISCUSSION

Regions in factor VIII containing high concentrations of negative charge density neighbor each of the three scissile bonds cleaved by thrombin during procofactor activation. The roles for these sites in the activation mechanism are poorly understood. In this report, we present direct evidence for the contribution of select acidic residues present within residues 717−725 in the a2 segment following the A2 domain in facilitating thrombin-catalyzed cleavages at Arg372 and Arg1689, as well as indirect evidence for a contribution to cleavage at Arg740. Based upon evaluation of point mutations made in this region, residues Glu724 and Asp725 and, to lesser extents, Glu720 and Asp721 appear to directly participate in interactions with thrombin. On the other hand, three Tyr residues, shown to be sulfated and localized within this segment, do not appear to appreciably contribute to the catalytic mechanism.

Replacement of the acidic residues in the 717−725 segment yielded factor VIII variants showing modest reductions in specific activity. In the case of these residues, the activity changes paralleled observed rate reductions in thrombin-catalyzed cleavage and consequent activation of factor VIII. For example, replacement of Glu717 with Ala showed minimal effects on specific activity and rates for proteolysis/procofactor activation, while the point mutations, Glu724Ala and Asp725Ala, were more severely affected in these parameter values, and the two double mutations at residues Glu720Ala/Asp721Ala and Glu724Ala/Asp725Ala showed the greatest parameter value reductions. These observations suggest a relationship between activity and activation rate, such that reductions in the latter impact factor VIII activity. Consistent with this conclusion is the similarity in V_{max} values for factor Xa generation for wild-type factor VIII and all of the acidic residue variants tested, indicating that once activated, full cofactor activity is expressed independent of mutations that reduce negative charge density within this segment of a2. The importance of this charge density is further indicated by the point mutation Glu720Lys, where this charge reversal mutation in factor VIII results in a mild hemophilia A phenotype (*27*).

Several studies have examined the contribution of the a2 region separating the A2 and B domains to factor VIII function and in particular thrombin interactions with the procofactor. This region represents a major epitope for inhibitor antibodies directed against factor VIII (*28*) that were shown to interfere with procofactor activation by thrombin (*28, 29*). Furthermore, a recombinant B-domainless factor VIII lacking residues 713−740 showed an ∼10-fold reduction in the second-order rate constant for thrombin-catalyzed activation of factor VIII (*14*). Moreover, that study revealed modest reductions in the rate for thrombin cleavage of heavy chain but significant (∼10-fold) rate reductions in light chain

Table 3: Rates of Subunit Generation for Wild-Type and Mutant Factor VIII at 37 °C and 140 mM NaCl[a]

factor VIII	rate of A1 subunit generation [nM A1 min^{-1}> (nM IIa)$^{-1}$]	rate of A2 subunit generation [nM A2 min^{-1} (nM IIa)$^{-1}$]	rate of A3-C1-C2 subunit generation [nM A3-C1-C2 min^{-1} (nM IIa)$^{-1}$]
wild type	53.8 ± 1.4	62.9 ± 5.1	304 ± 24
E720/721A	18.7 ± 1.9	14.2 ± 1.1	15.1 ± 2.5
E724/725A	10.0 ± 0.6	8.0 ± 1.5	8.2 ± 0.9

[a] Subunit generation rates for the A1, A2, and A3-C1-C2 subunits by thrombin cleavage of wild-type and mutant factor VIII were estimated by nonlinear regression analysis of the data shown in Figure 5. Experiments were performed at least three separate times, and average values are shown.

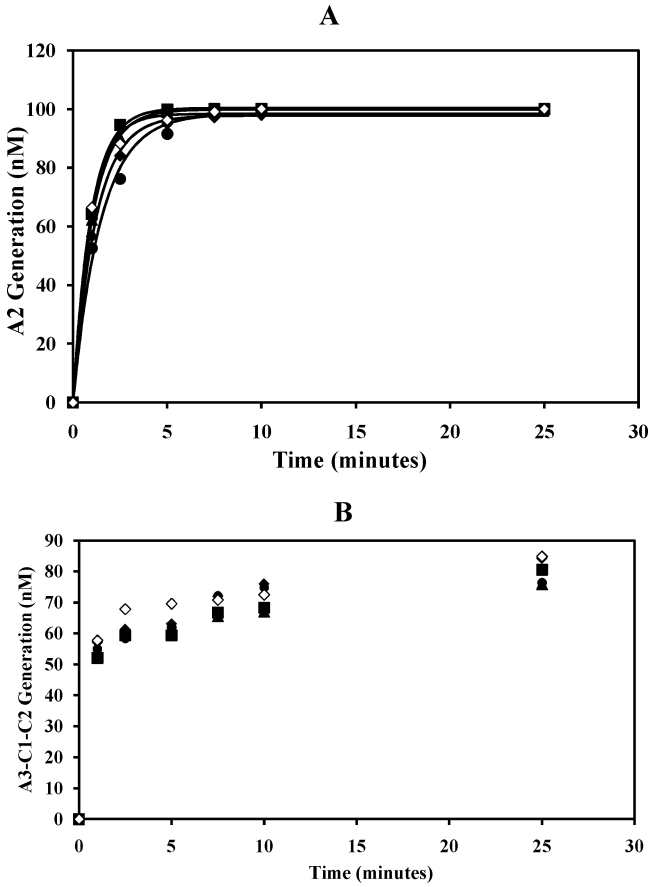

FIGURE 6: Western blot analysis of tyrosine sulfation mutants. Panel A: Recombinant wild-type and mutant factor VIII (100 nM) were reacted with thrombin (2.5 nM) for the indicated times and subjected to SDS−PAGE and blotting using the anti-A2 antibody (see Supporting Information for blots). Symbols: ◆, wild type; ●, Tyr718Phe; ■, Tyr719Phe; ▲, Tyr723Phe; ◇, Tyr718Phe/Tyr719Phe/Tyr723Phe. Panel B: The wild-type and mutant factor VII reactions were repeated as described above except blotting with the anti-A3 antibody (see Supporting Information for blots). Symbols: ◆, wild type; ●, Tyr718Phe; ■, Tyr719Phe; ▲, Tyr723Phe; ◇, Tyr718Phe/Tyr719Phe/Tyr723Phe.

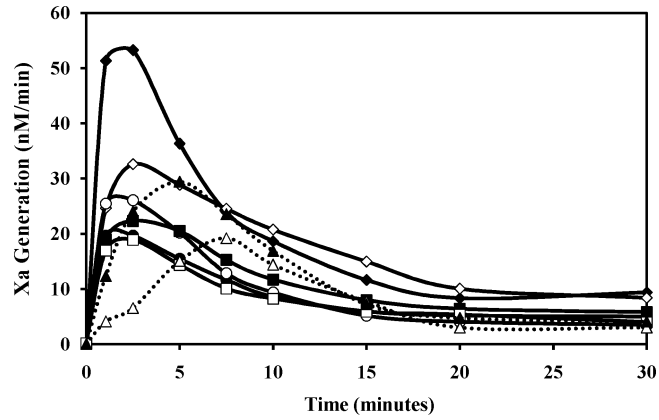

FIGURE 7: Activation of wild-type and mutant factor VIII by thrombin. Recombinant factor VIII WT and mutants (1 nM) were reacted with thrombin (0.05 nM) for the indicated times. Thrombin was inactivated by addition of hirudin (0.1 unit/mL), and factor VIIIa was reacted with factor IXa (20 nM) and phospholipid vesicles (10 μM). Factor Xa generation was initiated by addition of factor X (300 nM) as described in Materials and Methods. Symbols: ◆, wild type; ◇, Asp717Ala; ●, Glu720Ala; ○, Asp721Ala; ■, Glu724Ala; □, Asp725Ala; ▲, Glu720Ala/Asp721Ala Δ, Glu724Ala/Asp725Ala. Experiments were performed at least three separate times, and average values are shown. Continuous lines represent the single mutants while the double mutants are represented by the dotted lines.

Table 4: Kinetic Parameters of Wild-Type and Mutant Factor VIII[a]

factor VIII	K_m(app) (nM)	V_{max} for Xa generation (nM min^{-1})
wild type	32 ± 5.7	276 ± 21
E720A	56 ± 7.8	308 ± 25
D721A	43 ± 9.7	257 ± 20
E724A	135 ± 16	277 ± 9.6
D725A	173 ± 8.9	259 ± 14
E720A/D721A	78 ± 13	269 ± 26
E724A/D725A	230 ± 25	248 ± 9.2

[a] Kinetic parameters were determined from initial rate data using the Michaelis−Menten equation and nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown.

cleavage. These observations parallel our results showing that mutations within the 717−725 segment affect all three P1 sites in factor VIII with primary effect at the Arg1689 site. Thus we speculate that loss of this segment in the 713−740 deletion was in large part responsible for the observations made in the earlier study.

While negative charge density within residues 717−725 appears to be important in regulating catalysis by thrombin, the charge contribution appears asymmetric with some residues showing more pronounced effects than others. Mutation of residues Glu724 and Asp725 showed the most detrimental effects of the five individual acidic residue point mutations. This observation was based upon apparent rates for thrombin-catalyzed cleavage at each of the three scissile bonds in factor VIII as well as generation of factor VIIIa cofactor activity. Furthermore, these point mutations showed ~4−5-fold increases in the K_m for procofactor activation as compared with wild type. These results suggested a possible greater contribution of residues 724 and 725 to a thrombin exosite-dependent interaction than observed for the more N-terminal residues in the 717−725 segment. This differential regulation mechanism was compatible with results from Myles et al. (*16*) that showed thrombin anion-binding exosite mutants possessing varying effects on proteolysis of factor VIII.

Interestingly, thrombin activation of factor VIII did not appear to be affected by the negatively charged sulfated Tyr residues within the 717−725 segment. Tyrosine sulfation at

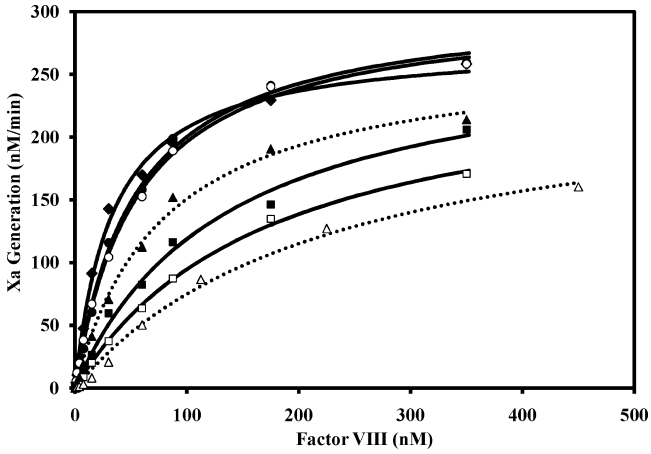

FIGURE 8: Kinetics of thrombin activation of wild-type and double mutant factor VIII. Various concentrations of recombinant wild-type and mutant factor VIII were reacted with thrombin (0.05 nM) for 15 s. Thrombin was inactivated by addition of hirudin (0.1 unit/mL) in the presence of phospholipid vesicles (10 μM), and factor Xa generation was initiated by addition of factor X (300 nM) and factor IXa (20 nM) as described in Materials and Methods. Symbols: ◆, wild type; ●, Glu720Ala; ○, Asp721Ala; ■, Glu724Ala; □, Asp725Ala; ▲, Glu720Ala/Asp721Ala; Δ, Glu724Ala/Asp725Ala. Initial rates of factor Xa generation are plotted as a function of factor VIII concentration and fitted to the Michaelis−Menten equation by nonlinear least squares regression. Experiments were performed at least three separate times, and average values are shown. Continuous lines represent the single mutants while the double mutants are represented by the dotted lines.

residues at 718, 719, and 723 in factor VIII was demonstrated in CHO cells by biosynthetic [^{35}S]sulfate labeling (*24*). Sulfation at these residues in BHK cell-expressed factor VIII was confirmed using amino acid composition analysis and liquid chromatography electrospray ionization tandem mass spectrometry (*25*). Although sulfation of specific Tyr residues is required for full factor VIII activity (*24*), the mechanism by which these modified residues in the a2 region contribute to function is not known. A previous study utilizing a transiently transfected triple mutant of Tyr718Phe/Tyr719Phe/Tyr723Phe revealed a modest reduction in specific activity (~2-fold that of wild-type factor VIII) (*26*). Western blotting of these tyrosine sulfation mutants (0.25 μg/mL) reacted with a molar excess of thrombin (0.125 μg/mL) revealed the rate of A2 subunit generation was somewhat reduced whereas no effects on generation of A1 or A3-C1-C2 subunits were observed, indicating only the P1 Arg740 site was affected. Our results are consistent with those of the earlier study and indicate no significant effects of these putative sulfated Tyr residues on thrombin cleavage of factor VIII. Thus, the modest reduction in activity observed for these mutations is likely the result of a defect in (a) function(s) other than those related to procofactor activation.

The A2 subunit of factor VIIIa (residues 372−740) possesses regions of high negative charge density at both its N-terminus (^{389}EEEDWD^{394}) and C-terminus (^{717}DYYEDSYED^{725}). In an earlier report, Nogami et al. (*17*) suggested that binding of thrombin to the isolated A2 subunit (residues 373−740) of factor VIIIa was primarily anion-binding exosite II-dependent. That conclusion was based upon the failure of hirudin to inhibit binding, whereas heparin yielded ~60−80% inhibition of thrombin binding to the isolated A2 subunit. Furthermore, FRET analysis indicated similar affinity values for thrombin and the isolated A2 subunit, as determined by using reactions conditions yielding slow, fast, and slow/fast thrombin forms suggesting that this interaction was independent of occupancy of the Na^+ binding site in thrombin. This observation was consistent with the lack of appreciable effect of NaCl concentration on the relative rate reductions in factor VIIIa subunit generation observed for the double mutants compared with wild-type factor VIII.

The above study by Nogami et al. (*17*) also showed markedly reduced rates of thrombin cleavage at Arg740, but not at Arg372 for an Asp392Ala/Asp394Ala double mutation in factor VIII. While cleavage at the light chain appeared unaffected for this variant, this rate was visualized by loss of blotting reactivity using an a3 segment-specific monoclonal antibody. However, the persistence of an A2-light chain adduct during the reaction time course suggested impaired cleavage of both Arg740 and Arg1689 in the single chain form of this factor VIII variant. Observations in the present study show that mutations within the C-terminal acidic region impair all cleavage rates, with those at Arg740 and Arg1689 sites appearing most severely affected. Results with the Glu724Ala/Asp725Ala double mutant show that rates for A2 and A3-C1-C2 subunit generation are reduced ~10- and ~30-fold, while the rate for A1 subunit generation is reduced ~4-fold compared with WT factor VIII. It is important to note that A2 subunit is derived from both the heavy chain of the factor VIII heterodimer, where its rate of appearance should equal that of the A1 subunit, and from single chain factor VIII, where cleavages at both Arg740 and Arg372 are required to yield the A2 product. Thus a greater reduction in the rate of A2 generation compared with A1 generation in the variant necessarily indicates a significant reduction in the rate of cleavage at Arg740. The similarity in the effects of mutations in both the N- and C-terminal acidic regions on products generated by thrombin suggests that residues in both regions may contribute to a single exosite II-interactive site in the folded protein.

The homologous procofactor, factor V, is activated by thrombin-catalyzed cleavages with sequential proteolysis at Arg709, Arg1018, and Arg1545 to yield the factor Va heterodimer (*30, 31*). As in factor VIII, these reactions are exosite-driven (*32*) and rely upon clusters of acidic residues in the procofactor for efficient interaction with the proteinase. One such region in factor V is represented by residues 695−699 (DYDYQ) located at the C-terminal region of the heavy chain in an homologous region to the 717−725 sequence in factor VIII. An earlier report showed that a factor V variant where the two Asp residues were replaced with Lys and the two Tyr replaced with Phe was partially resistant to activation by thrombin (*33*). This result, taken together with the results from the current study, underscores the importance of selected residues contributing to high negative charge density localized within the A2-B domain junction in the mechanism for thrombin-catalyzed activation of the coagulation procofactors.

ACKNOWLEDGMENT

We thank Pete Lollar and John Healey for the gift of the factor VIII cloning and expression vectors, Zaverio Ruggeri and Lisa Regan for the C5 and 2D2 monoclonal antibodies, respectively, and Qian Zhou for excellent technical assistance.

SUPPORTING INFORMATION AVAILABLE

Western blot time courses of the acidic and tyrosine sulfation factor VIII variants following reaction with thrombin and using the A2- and A3-domain specific antibodies (Figures 1, 2, and 3). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wood, W. I., Capon, D. J., Simonsen, C. C., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hollingshead, P., and Wion, K. L. (1984) Expression of Active Human Factor VIII from Recombinant DNA Clones. *Nature 312*, 330–337.
2. Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., and Harkins, R. N. (1984) Structure of Human Factor VIII. *Nature 312*, 337–342.
3. Fay, P. J. (2004) Activation of Factor VIII and Mechanisms of Cofactor Action. *Blood Rev. 18*, 1–15.
4. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Surface-Dependent Reactions of the Vitamin K-Dependent Enzyme Complexes. *Blood 76*, 1–16.
5. Eaton, D., Rodriguez, H., and Vehar, G. A. (1986) Proteolytic Processing of Human Factor VIII. Correlation of Specific Cleavages by Thrombin, Factor Xa, and Activated Protein C with Activation and Inactivation of Factor VIII Coagulant Activity. *Biochemistry 25*, 505–512.
6. Parker, E. T., Pohl, J., Blackburn, M. N., and Lollar, P. (1997) Subunit Structure and Function of Porcine Factor Xa-Activated Factor VIII. *Biochemistry 36*, 9365–9373.
7. Pieters, J., Lindhout, T., and Hemker, H. C. (1989) In Situ-Generated Thrombin is the Only Enzyme that Effectively Activates Factor VIII and Factor V in Thromboplastin-Activated Plasma. *Blood 74*, 1021–1024.
8. Fay, P. J., Haidaris, P. J., and Smudzin, T. M. (1991) Human Factor VIIIa Subunit Structure. Reconstruction of Factor VIIIa from the Isolated A1/A3-C1-C2 Dimer and A2 Subunit. *J. Biol. Chem. 266*, 8957–8962.
9. Lollar, P., and Parker, E. T. (1991) Structural Basis for the Decreased Procoagulant Activity of Human Factor VIII Compared to the Porcine Homolog. *J. Biol. Chem. 266*, 12481–12486.
10. Newell, J. L., and Fay, P. J. (2007) Proteolysis at Arg740 Facilitates Subsequent Bond Cleavages during Thrombin-Catalyzed Activation of Factor VIII. *J. Biol. Chem. 282*, 25367–25375.
11. Fay, P. J., Mastri, M., Koszelak, M. E., and Wakabayashi, H. (2001) Cleavage of Factor VIII Heavy Chain is Required for the Functional Interaction of A2 Subunit with Factor IXa. *J. Biol. Chem. 276*, 12434–12439.
12. Lollar, P., Hill-Eubanks, D. C., and Parker, C. G. (1988) Association of the Factor VIII Light Chain with Von Willebrand Factor. *J. Biol. Chem. 263*, 10451–10455.
13. Regan, L. M., and Fay, P. J. (1995) Cleavage of Factor VIII Light Chain is Required for Maximal Generation of Factor VIIIa Activity. *J. Biol. Chem. 270*, 8546–8552.
14. Donath, M. J., Lenting, P. J., Van Mourik, J. A., and Mertens, K. (1996) Kinetics of Factor VIII Light-Chain Cleavage by Thrombin and Factor Xa. A Regulatory Role of the Factor VIII Heavy-Chain Region Lys713-Arg740. *Eur. J. Biochem. 240*, 365–372.
15. Esmon, C. T., and Lollar, P. (1996) Involvement of Thrombin Anion-Binding Exosites 1 and 2 in the Activation of Factor V and Factor VIII. *J. Biol. Chem. 271*, 13882–13887.
16. Myles, T., Yun, T. H., and Leung, L. L. (2002) Structural Requirements for the Activation of Human Factor VIII by Thrombin. *Blood 100*, 2820–2826.
17. Nogami, K., Zhou, Q., Myles, T., Leung, L. L., Wakabayashi, H., and Fay, P. J. (2005) Exosite-Interactive Regions in the A1 and A2 Domains of Factor VIII Facilitate Thrombin-Catalyzed Cleavage of Heavy Chain. *J. Biol. Chem. 280*, 18476–18487.
18. Saenko, E. L., Shima, M., Gilbert, G. E., and Scandella, D. (1996) Slowed Release of Thrombin-Cleaved Factor VIII from Von Willebrand Factor by a Monoclonal and a Human Antibody is a Novel Mechanism for Factor VIII Inhibition. *J. Biol. Chem. 271*, 27424–27431.
19. Casillas, G., Simonetti, C., and Pavlovsky, A. (1971) Artificial Substrate for the Assay of Factor V and VIII. *Coagulation 4*, 107–111.
20. Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Phospholipid Vesicle Formation and Transmembrane Protein Incorporation using Octyl Glucoside. *Biochemistry 20*, 833–840.
21. Jenkins, P. V., Freas, J., Schmidt, K. M., Zhou, Q., and Fay, P. J. (2002) Mutations Associated with Hemophilia A in the 558−565 Loop of the Factor VIIIa A2 Subunit Alter the Catalytic Activity of the Factor Xase Complex. *Blood 100*, 501–508.
22. Wakabayashi, H., Freas, J., Zhou, Q., and Fay, P. J. (2004) Residues 110−126 in the A1 Domain of Factor VIII Contain a Ca^{2+} Binding Site Required for Cofactor Activity. *J. Biol. Chem. 279*, 12677–12684.
23. Lollar, P., Fay, P. J., and Fass, D. N. (1993) Factor VIII and Factor VIIIa. *Methods Enzymol. 222*, 128–143.
24. Pittman, D. D., Wang, J. H., and Kaufman, R. J. (1992) Identification and Functional Importance of Tyrosine Sulfate Residues within Recombinant Factor VIII. *Biochemistry 31*, 3315–3325.
25. Severs, J. C., Carnine, M., Eguizabal, H., and Mock, K. K. (1999) Characterization of Tyrosine Sulfate Residues in Antihemophilic Recombinant Factor VIII by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry and Amino Acid Analysis. *Rapid Commun. Mass Spectrom. 13*, 1016–1023.
26. Michnick, D. A., Pittman, D. D., Wise, R. J., and Kaufman, R. J. (1994) Identification of Individual Tyrosine Sulfation Sites within Factor VIII Required for Optimal Activity and Efficient Thrombin Cleavage. *J. Biol. Chem. 269*, 20095–20102.
27. Kemball-Cook, G., Tuddenham, E. G., and Wacey, A. I. (1998) The Factor VIII Structure and Mutation Resource Site: HAMSTeRS Version 4. *Nucleic Acids Res. 26*, 216–219.
28. Leyte, A., Mertens, K., Distel, B., Evers, R. F., De Keyzer-Nellen, M. J., Groenen-Van Dooren, M. M., De Bruin, J., Pannekoek, H., Van Mourik, J. A., and, and Verbeet, M. P. (1989) Inhibition of Human Coagulation Factor VIII by Monoclonal Antibodies. *Biochem. J. 263*, 187–194.
29. van den Brink, E. N., Turenhout, E. A., Bank, C. M., Fijnvandraat, K., Peters, M., and Voorberg, J. (2000) Molecular Analysis of Human Anti-Factor VIII Antibodies by V Gene Phage Display Identifies a New Epitope in the Acidic Region Following the A2 Domain. *Blood 96*, 540–545.
30. Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., and Mann, K. G. (1987) Complete cDNA and Derived Amino Acid Sequence of Human Factor V. *Proc. Natl. Acad. Sci. U.S.A. 84*, 4846–4850.
31. Dharmawardana, K. R., Olson, S. T., and Bock, P. E. (1999) Role of Regulatory Exosite I in Binding of Thrombin to Human Factor V, Factor Va, Factor Va Subunits, and Activation Fragments. *J. Biol. Chem. 274*, 18635–18643.
32. Myles, T., Yun, T. H., Hall, S. W., and Leung, L. L. (2001) An Extensive Interaction Interface between Thrombin and Factor V is Required for Factor V Activation. *J. Biol. Chem. 276*, 25143–25149.
33. Kalafatis, M., Beck, D. O., and Mann, K. G. (2003) Structural Requirements for Expression of Factor Va Activity. *J. Biol. Chem. 278*, 33550–33561.

BI8007824