Factor VIII accelerates proteolytic cleavage of von Willebrand factor by ADAMTS13

Wenjing Cao*, Sriram Krishnaswamy†, Rodney M. Camire†, Peter J. Lenting‡§, and X. Long Zheng*¶

Departments of *Pathology and Laboratory Medicine and †Pediatrics, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA 19104; ‡Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, 3508 GA, Utrecht, The Netherlands; and §Department of Protein Discovery, Crucell Holland B.V., 2301 CA, Leiden, The Netherlands

Edited by Mark T. Groudine, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved March 27, 2008 (received for review February 28, 2008)

Proteolytic processing of von Willebrand factor (VWF) by ADAMTS13 metalloproteinase is crucial for normal hemostasis. *In vitro*, cleavage of VWF by ADAMTS13 is slow even at high shear stress and is typically studied in the presence of denaturants. We now show that, under shear stress and at physiological pH and ionic strength, coagulation factor VIII (FVIII) accelerates, by a factor of ≈10, the rate of specific cleavage at the Tyr^{1605}–Met^{1606} bond in VWF. Multimer analysis reveals that FVIII preferentially accelerates the cleavage of high-molecular-weight multimers. This rate enhancement is not observed with VWF predenatured with 1.5 M guanidine. The ability of FVIII to enhance VWF cleavage by ADAMTS13 is rapidly lost after pretreatment of FVIII with thrombin. A FVIII derivative lacking most of the B domain behaves equivalently to full-length FVIII. In contrast, a derivative lacking both the B domain and the acidic region a3 that contributes to the high-affinity interaction of FVIII with VWF exhibits a greatly reduced ability to enhance VWF cleavage. Our data suggest that FVIII plays a role in regulating proteolytic processing of VWF by ADAMTS13 under shear stress, which depends on the high-affinity interaction between FVIII and its carrier protein, VWF.

thrombotic thrombocytopenic purpura | von Willebrand factor cleaving metalloprotease

Normal hemostasis requires the proteolytic processing of newly synthesized and secreted von Willebrand factor (VWF) in plasma by the metalloproteinase ADAMTS13. This process reduces VWF multimer size by cleavage at the Tyr^{1605}–Met^{1606} bond in the A2 subunit (1). Defective proteolytic processing of VWF multimers due to hereditary and/or acquired deficiency of ADAMTS13 activity results in the accumulation of "unusually large" multimers of VWF in plasma and disseminated microthrombi (2). These are characteristic pathological features of thrombotic thrombocytopenic purpura.

ADAMTS13 cleaves VWF in a relatively inefficient way, and its preference for cleavage of the larger multimers is considered to lie in their greater susceptibility to deformation under shear stress or under flow while tethered to platelets (3). Previous studies have used a variety of manipulations to enhance cleavage including the use of "low concentrations" of urea (4) or guanidine hydrochloride (5) and prolonged incubation of denatured VWF with ADAMTS13 at low ionic strength, alkaline pH, and high concentrations of various divalent cations such as Ba^{2+}, Ca^{2+}, and Zn^{2+} (1, 4, 6). These strategies are reminiscent of the extreme conditions used for observable cleavage of the coagulation zymogens in the absence of membranes and the appropriate cofactors that are now known to be essential for their efficient activation (7). Only very small enhancing effects of heparin and glycoprotein Ib on cleavage of denatured VWF have been reported previously (8). Thus, efficient processing of VWF by ADAMTS13 under more physiological conditions may be further regulated by a cofactor or by accessory components that are yet to be recognized.

An obvious candidate for this role is coagulation factor VIII (FVIII), which binds VWF with subnanomolar affinity (9). FVIII circulates in blood at ≈0.3–0.7 nM, essentially fully bound to VWF ([subunits] = 40 nM), which functions as a carrier protein, prolonging its half-life and protecting it from proteolytic inactivation (9, 10). Proteolytic activation by thrombin releases the resultant FVIIIa from VWF, allowing it to function in the intrinsic pathway of blood coagulation (9). Although each VWF subunit can bind one molecule of FVIII, at physiological concentrations approximately one FVIII is bound per 60–130 VWF monomers (11, 12). Thus, in blood, the fraction of VWF monomers saturated with FVIII is small, and the likelihood of encountering FVIII bound to any one multimer is expected to increase with multimer length (12).

In the present study we have used mechanical shear stress to investigate VWF processing by ADAMTS13 in the absence of denaturants and in buffers at physiological pH and ionic strength. Our work demonstrates that FVIII accelerates proteolytic cleavage of VWF by ADAMTS13 and suggests that the ability of FVIII to bind VWF plays an important role in its ability to function as a cofactor for VWF processing under physiological conditions.

Results

FVIII Enhances Proteolytic Cleavage of VWF by ADAMTS13 Under Shear Stress. Purified plasma-derived VWF (pVWF; 37.5 μg/ml or 150 nM) was incubated with recombinant ADAMTS13 (50 nM) for 3 min under constant vortexing in the absence and in the presence of various concentrations (0–40 nM) of recombinant FVIII. Proteolysis of VWF was detected by the appearance of an immunoreactive fragment [M_r = 350,000 (350K)] representing a disulfide bond-linked dimer resulting from cleavage by ADAMTS13 after Tyr^{1605} in two adjacent subunits within the VWF multimer (1). Cleavage rate and product formation were increased with increasing concentrations of FVIII (Fig. 1). In multiple experiments, ADAMTS13 function, detected in this way, was enhanced by ≈10- to ≈12-fold in the presence of ≈10–20 nM FVIII (Fig. 1). The concentration of FVIII required for half-maximal enhancement in product formation was ≈3.0 nM (Fig. 1), indicating that these increases in product formation occur within the realm of the marginal fractional saturation of VWF monomers within the multimer by FVIII expected *in vivo* (12).

Enhanced VWF cleavage resulting from buffer artifacts could be excluded because FVIII had been repurified by cation exchange chromatography, dialyzed, and stored in assay buffer

Author contributions: W.C., S.K., R.M.C., and X.L.Z. designed research; W.C., S.K., R.M.C., and X.L.Z. performed research; P.J.L. contributed new reagents/analytic tools; W.C., S.K., R.M.C., and X.L.Z. analyzed data; and W.C., S.K., R.M.C., and X.L.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¶To whom correspondence should be addressed at: Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, University of Pennsylvania Medical Center, 816G Abramson Research Center, 3615 Civic Center Boulevard, Philadelphia, PA 19104. E-mail: zheng@email.chop.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801735105/DCSupplemental.

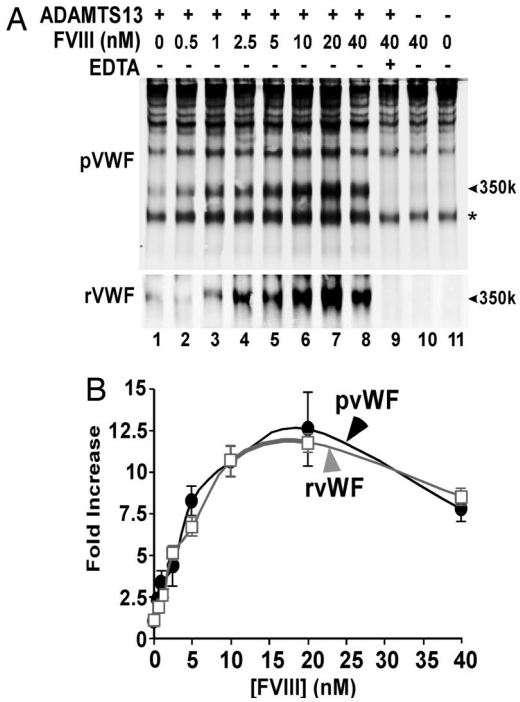

Fig. 1. FVIII enhances proteolytic cleavage of multimeric VWF by ADAMTS13 under shear stress. (*A*) pVWF or rVWF (150 nM) was incubated without (−) and with (+) ADAMTS13 (50 nM) in the absence (lane 1) and the presence (lanes 2–9) of the indicated concentrations of FVIII. Lane 9 contained 40 nM FVIII plus 20 mM EDTA. The 350K cleavage product was visualized by Western blot analysis after 3 min of vortexing. A nonspecific, preexisting band in the VWF preparations of unknown origin that also accumulates with proteolysis is denoted by an asterisk. (*B*) Increase in cleavage product detected relative to that observed in the absence of FVIII (Fold Increase) was determined by densitometry. Results represent the mean ± standard deviation of four independent experiments.

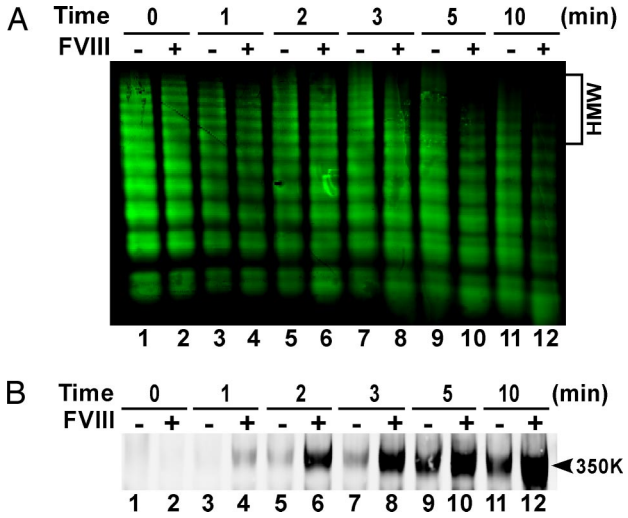

Fig. 2. FVIII preferentially accelerates cleavage of high-molecular-weight VWF by ADAMTS13 under shear stress. pVWF (150 nM) was incubated with recombinant ADAMTS13 (50 nM) in the absence (−) and presence (+) of 20 nM FVIII and vortexed at 2,500 rpm for the indicated times. Proteolysis was assessed by immunological detection of multimers (*A*) or the detection of the M_r = 350,000 fragment (*B*). HMW denotes high-molecular-weight multimers.

lacking BSA. Furthermore, immunoprecipitation with goat anti-FVIII IgG bound to protein G-Sepharose largely eliminated the rate-enhancing effects of FVIII added to the cleavage reaction (data not shown). Other control experiments established the lack of detectable cleavage after the addition of FVIII in the absence of ADAMTS13 (Fig. 1) or addition of EDTA (20 mM) to complete reaction mixtures (Fig. 1). These findings rule out some obvious trivial explanations for the observations.

VWF purified from plasma can contain as much as 1% (wt/wt) contaminating FVIII (10). The presence of endogenous contaminating FVIII or its proteolytic fragments could obscure the true extent to which added FVIII enhances VWF cleavage. This possibility was assessed by using purified recombinant VWF (rVWF) expressed in baby hamster kidney cells, and this rVWF was thus never previously exposed to detectable levels of FVIII. The results with rVWF were equivalent to those obtained by using pVWF. With rVWF as substrate, product formation was increased ≈10-fold in the presence of 20 nM FVIII with a half-maximal effect also observed at ≈3 nM (Fig. 1). Any possible endogenous FVIII in VWF purified from plasma is not functional in this assay, possibly owing to its inactivation or degradation during VWF purification.

FVIII Preferentially Accelerates Cleavage of High-Molecular-Weight VWF Multimers. Detection of the cleaved fragment (350K) facilitates "semiquantitative" and somewhat defined measurements of ADAMTS13 function (1). However, it is a potentially misleading measurement because product is detected only after cleavages in two adjacent VWF subunits that are not a requirement for the biologically relevant processing of VWF multimers by ADAMTS13. We assessed the VWF multimer distribution after digestion with ADAMTS13 in the absence and in the presence of 20 nM FVIII. Agarose gel electrophoresis and Western blot analysis revealed a dramatic reduction in high-molecular-weight multimers of VWF in the presence of FVIII (Fig. 2*A*). The degradation of high-molecular-weight VWF multimers was time-dependent and was also associated with an increase in formation of the degradation product (350K) (Fig. 2*B*). These findings indicate that the loss of the larger multimers results from proteolytic cleavage of VWF at the Tyr^{1605}–Met^{1606} bond by ADAMTS13 and not from nonspecific adsorption or aggregation-related depletion of multimers after their exposure to high shear.

Denaturation of VWF Abolishes FVIII Effects on ADAMTS13 Function. Pretreatment of VWF with 1.5 M guanidine increases its cleavage by ADAMTS13 when assessed at low ionic strength (1). To determine whether FVIII affects VWF proteolysis by ADAMTS13 under such conditions that are widely used to assess enzyme activity, increasing concentrations of FVIII were added to reaction mixtures containing guanidine-denatured VWF (150 nM) and recombinant ADAMTS13 (12.5 nM) in 50 mM Hepes (pH 7.5) and 50 mM NaCl at 37°C. Reaction progress was monitored at various times (0, 5, 10, 30, and 60 min) after initiation by immunodetection of the 350K cleavage product. No increase in cleavage product was detected at any time point in the presence of 20 nM FVIII (data not shown) or in the presence of increasing FVIII concentrations after a 1-h incubation (Fig. 3). Thus, FVIII does not play a role in enhancing the digestion of "unfolded" VWF by ADAMTS13.

Thrombin Activation of FVIII Modulates Its Role in Affecting VWF Proteolysis. Proteolytic activation of FVIII by thrombin is enhanced when it is bound to VWF (13, 14). The resulting heterotrimeric FVIIIa dissociates from VWF and exhibits labile procoagulant activity because of the rapid dissociation of the A2 subunit (9, 15, 16). FVIII was rapidly activated by the addition of high concentrations of thrombin followed by inhibition of thrombin with hirudin resulting in the quantitative formation of

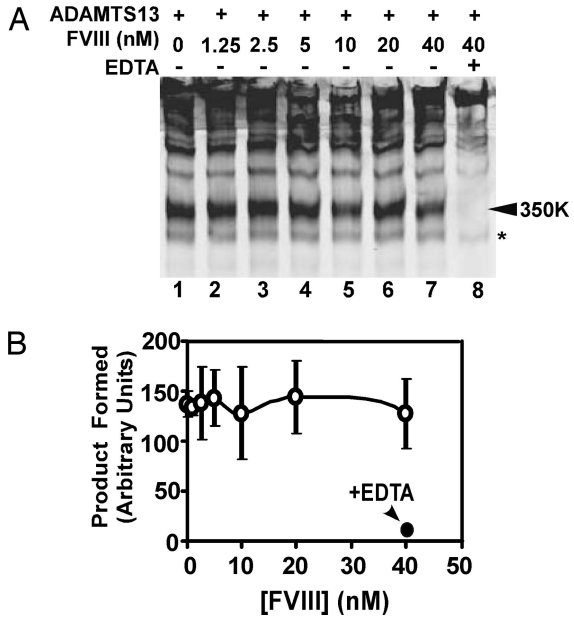

Fig. 3. FVIII has no effect on cleavage of denatured VWF under static conditions. (*A*) pVWF (150 nM) pretreated with guanidine was incubated for 1 h at 37°C with recombinant ADAMTS13 (12.5 nM) in the absence (lane 1) and the presence (lanes 2–7) of the indicated concentrations of FVIII. Lane 7 contained 40 nM FVIII plus 20 mM EDTA. Proteolysis was assessed by immunological detection of the 350K fragment. An asterisk indicates the preexisting band in the VWF preparation. (*B*) Dependence of product formation on the concentration of FVIII was determined by densitometry analysis and is presented as mean ± standard deviation of three experiments.

FVIIIa characterized by A1, A2, and A3–C1–C2 fragments (Fig. 4*A*; LC). At various times after activation, FVIIIa (20 nM) was added to reaction mixtures containing VWF (150 nM) and

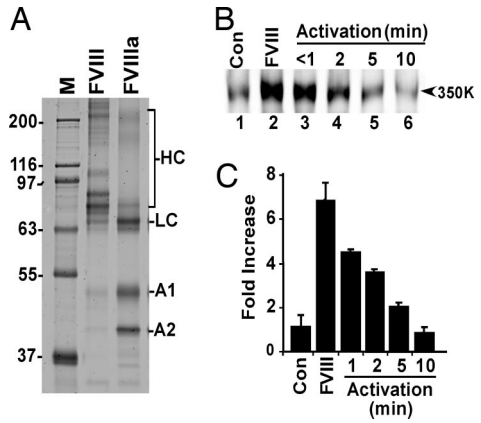

Fig. 4. Proteolytic activation alters FVIII effects on VWF cleavage by ADAMTS13 under shear stress. (*A*) SDS/PAGE analysis of purified FVIII (lane 2) and FVIIIa (lane 3) 30 s after incubation with thrombin. Protein bands were visualized by staining with SYPRO Ruby fluorescent dye. The first lane contains markers with the indicated molecular weights (× 10^3). HC, LC, A1, and A2 denote heavy chain, light chain, A1, and A2 fragments. (*B*) pVWF (150 nM) was incubated with recombinant ADAMTS13 (50 nM) under constant vortexing for 3 min in the absence (lane 1) and in the presence (lane 2) of 20 nM FVIII and at the indicated times after rapid activation of 20 nM FVIII with 20 nM human thrombin and quenched with 30 nM hirudin (lanes 4–6). VWF proteolysis was assessed by immunological detection of the M_r = 350,000 fragment. (*C*) Product formation relative to that observed in the presence of ADAMTS13 alone was determined by quantitative densitometry. Results are presented as mean ± standard deviation of three experiments.

recombinant ADAMTS13 (50 nM). The 350K cleavage product was detected after a 3-min incubation under constant vortexing. Enhanced product formation rapidly decreased to control levels with a half-life of ≈2 min (Fig. 4 *B* and *C*). Thus, activation of FVIII by thrombin and the dissociation of FVIIIa from VWF and/or dissociation of the A2 subunit eliminated its ability to enhance cleavage of VWF by ADAMTS13. This points to an additional mechanism that may play a role in regulating ADAMTS13-mediated VWF proteolysis during ongoing coagulation.

Binding of FVIII to VWF Correlates with Its Ability to Enhance VWF Cleavage. Two recombinant FVIII derivatives were used to investigate the relationship between its ability to bind VWF and the modulation of VWF cleavage by ADAMTS13. The control construct, the B-domainless FVIII-SQ, contained only 14 residues of the 909 residues in the B domain (Fig. 5*A*). The second B-domainless derivative, FVIII-2RKR, was designed with a PACE/furin site to allow secretion of a two-chain species lacking acidic region 3 at the N terminus of the light chain (Fig. 5*A*). As expected, SDS/PAGE analysis revealed that the light chain of construct FVIII-2RKR was slightly smaller than that of FVIII-SQ (Fig. 5*B*). Both FVIII-SQ and FVIII-2RKR are expected to exhibit procoagulant activity, whereas only FVIII-SQ but not FVIII-2RKR is expected to bind VWF with high affinity (9). Accordingly, the specific activity determined by activated thromboplastin time for FVIII-2RKR (35,000 units/mg) was roughly comparable to that of FVIII-SQ (10,000 ± 350 units/mg). FVIII-2RKR bound poorly to immobilized VWF in comparison to FVIII-SQ (Fig. 5*C*). This finding is in agreement with other studies implicating a role for acidic region 3 in the interaction of FVIII and VWF (13, 17). When assessed in assays for VWF cleavage, FVIII-SQ behaved equivalently to full-length FVIII yielding an ≈10-fold increase in VWF proteolysis by ADAMTS13 (Fig. 6). Half-maximal effects were observed with ≈2.5 nM FVIII-SQ, comparable to the findings with full-length FVIII (Fig. 6). These data suggest that most of the central B domain of FVIII is dispensable for its function in modulating VWF processing. In contrast, FVIII-2RKR failed, even at the highest concentration tested, to significantly enhance cleavage of VWF by ADAMTS13 (Fig. 6), suggesting that the high-affinity binding interaction between FVIII and VWF plays an important role in the ability of FVIII to accelerate ADAMTS13-mediated VWF cleavage.

FVIII also Interacts with ADAMTS13. We used measurements of peptidyl substrate cleavage by ADAMTS13 to assess whether FVIII could directly bind the proteinase and modulate its activity. This approach was pursued because the VWF fragments used in the peptidyl assay are not expected to bind FVIII. FVIII, FVIII-SQ, and FVIII-2RKR increased the initial rate of cleavage of FRETS-VWF73 [supporting information (SI) Fig. S1] and GST-VWF73 (data not shown) by a factor of 2 or 3. The data raise the possibility that FVIII and its derivatives may interact with ADAMTS13 and modulate its activity, albeit in a small way. This possibility was further explored by surface plasmon resonance measurements with immobilized full-length ADAMTS13 (Fig. S2). All three FVIII derivatives bound ADAMTS13 with apparently rapid on rate and off rate (data not shown). Equilibrium dissociation constants were estimated from the dependence of the plateau signal on the concentration of FVIII derivative injected (Fig. S2). Analysis according to the binding of FVIII to equivalent and noninteracting sites with a site concentration well below K_d yielded equilibrium dissociation constants ranging from 20 nM to 80 nM for the three FVIII derivatives (Fig. S2). These affinities are modest in comparison to the concentrations of FVIII (0.3–0.7 nM) and ADAMTS13 (5–7 nM) in plasma. Taken together with the small enhancement

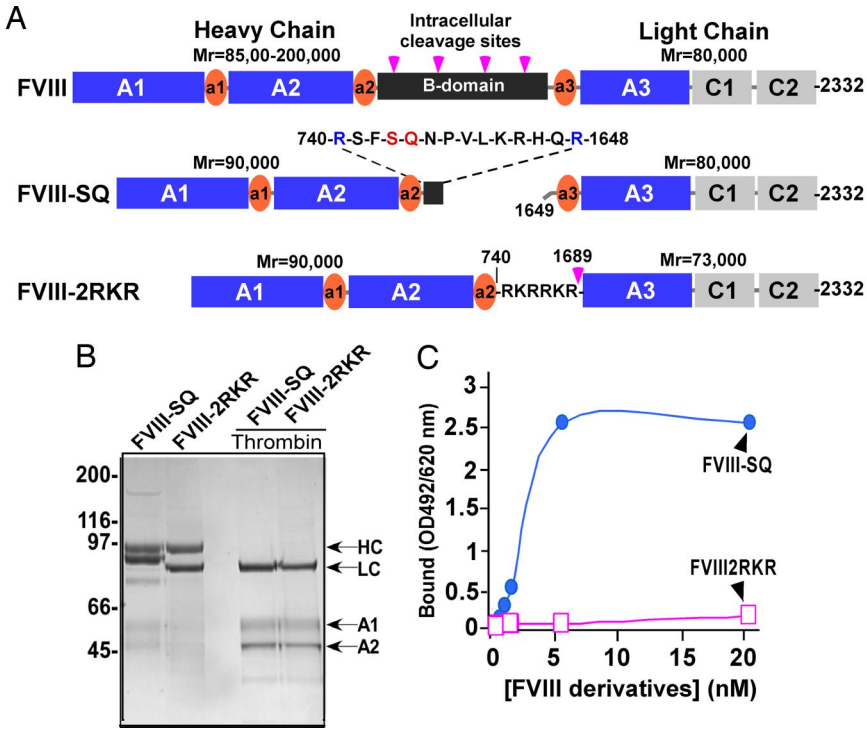

Fig. 5. Properties of FVIII derivatives. (*A*) Schematic representation of the domain structure of FVIII and derivatives. The heavy chain composed of A1–A2 domains is linked to a heterogeneously processed B domain of variable length. The light chain is composed of A3–C1–C2 domains. The three acidic regions are denoted as a1, a2, and a3. FVIII-SQ is secreted as a two-chain molecule in which the heavy chain contains 14 residues of the B domain. FVIII-2RKR is similar to FVIII-SQ, but it lacks a3. (*B*) FVII-SQ and FVIII-2RKR before and after activation by thrombin were analyzed by SDS/PAGE and visualized by staining with Coomassie blue. HC, LC, A1, and A2 denote the positions of the heavy and light chains and A1 and A2 domains. The first lane contains molecular weight markers with the indicated molecular weights ($\times 10^3$). (*C*) Binding of increasing concentrations of FVIII-SQ or FVIII-2RKR to immobilized VWF detected in an ELISA format.

in the rate of peptidyl cleavage, our data suggest that direct interactions between FVIII and ADAMTS13, independent of VWF, likely contribute in a minor way to the overall rate-enhancing effect on proteolytic cleavage of the macromolecular VWF substrate by ADAMTS13.

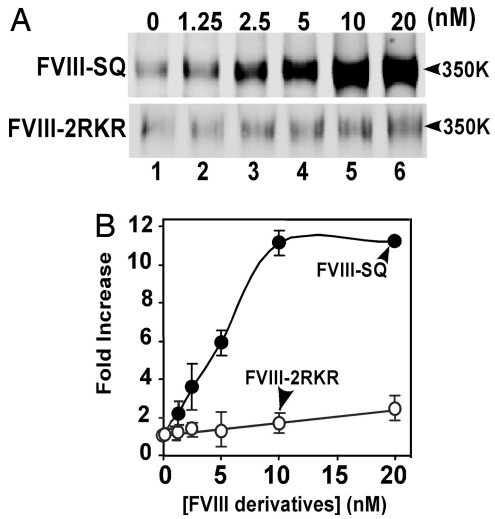

Fig. 6. FVIII-SQ but not FVIII-2RKR enhances proteolytic cleavage of VWF by ADAMTS13 under shear stress. (*A*) pVWF (150 nM) was incubated with recombinant ADAMTS13 (50 nM) in the presence of the indicated concentrations of FVIII-SQ or FVIII-2RKR for 3 min under vortexing at 2,500 rpm. Proteolysis was assessed by immunological detection of the M_r = 350,000 fragment (*A*) followed by densitometry analysis of product formed normalized to the product observed in the absence of FVIII derivative (*B*). Means ± standard deviations from three experiments are illustrated.

Discussion

Cofactor proteins play a fundamental role in enhancing proteinase function in the coagulation cascade. The present work was stimulated by the striking similarities in the extreme conditions used to observe detectable cleavage of VWF by ADAMTS13 and earlier work with coagulation proteinases before the essential contributions of cofactors and membranes were fully appreciated (7).

A search for cofactors that could modulate VWF processing by ADAMTS13 has been hindered by the lack of appropriate assays. The requirement for denaturants such as urea and guanidine and the use of buffers at nonphysiological pH and ionic strength could all obscure contributions of other components to proteinase function. The development of a simple shear stress-based assay (18) has provided an opportunity to investigate cofactor-dependent regulation of ADAMTS13 using the native macromolecular substrate and buffer conditions that are more consistent with the physiologic milieu. We show that FVIII accelerates the action of ADAMTS13 on VWF at concentrations that are consistent with the expected marginal saturation of VWF monomers with FVIII in blood. It is also not surprising that these enhancing effects of FVIII are completely obscured after the use of guanidine to denature the substrate.

The high-affinity interaction between FVIII and VWF evidently plays a key role in the ability of FVIII to enhance VWF proteolysis by ADAMTS13 under shear stress. This conclusion follows from the inability of FVIII-2RKR, lacking acidic region 3, to bind with high affinity to VWF or to enhance VWF proteolysis. Alternatively, we also present evidence for the ability

BIOCHEMISTRY

of FVIII to bind ADAMTS13 with modest affinity and produce a small increase in catalytic activity. It is clear that this is unlikely to represent the primary mechanism underlying FVIII function in this system particularly because both FVIII and FVIII-2RKR have equivalent effects on the activity of ADAMTS13 toward peptidyl substrates. We suspect that these observations reflect the features of a three-body problem wherein coupled interactions among FVIII, ADAMTS13, and VWF poise the proteinase on the multimer for enhanced cleavage at Tyr^{1605}. However, it is also possible that the enhancing effects of FVIII reflect its ability to bind VWF and somehow change its conformation and/or its susceptibility to deformation by shear stress.

The observed increase in product formation, resulting from the apparent ability of FVIII to function as a cofactor for VWF cleavage, pales in comparison to the increases in rate associated with cofactor function in the blood coagulation reactions (7). It is possible that the 10-fold increase in rate, afforded by FVIII, is sufficient to play an important role in VWF multimer processing in blood where FVIII is constitutively bound to VWF and ADAMTS13 circulates as an active proteinase. However, it is more likely that the true magnitude of the rate-enhancing effect is obscured by the complexity of the measurement that relies on immunodetection, with obvious associated problems, of a 350K cleavage product produced only upon cleavage in two adjacent subunits within the multimer. Measurements that rely on the formation of product produced in this way are more than likely to greatly underestimate the rate of the individual cleavage events and the rate at which VWF multimer size is reduced by ADAMTS13.

Accordingly, the effects of FVIII appear far more dramatic when assessed by multimer distributions where the presence of FVIII leads to a selective enhancement in consumption of the larger multimers of VWF. This effect, observed in the absence of denaturants, and rationalized on the basis of the very minimal fractional saturation of VWF with FVIII, provides a potentially cogent explanation for the selective cleavage of unusually large VWF multimers by ADAMTS13 *in vivo*. This potential explanation predicts impaired multimer processing in patients with severe hemophilia A (grossly deficient in FVIII) or excessive proteolysis in patients receiving high doses of FVIII.

It has previously been reported that VWF antigen and ristocetin cofactor activity are elevated (≈2-fold) in severe hemophilia A patients compared with healthy controls (19). In addition, acquired von Willebrand disease has been reported in a patient receiving prolonged infusion of a high dose of recombinant FVIII after surgery (20), although other causes of VWF depletion cannot be ruled out. We are unaware of reports documenting a disproportionate increase in large VWF multimers in severe hemophilia A patients. The reasons for this may include (*i*) lack of quantitative methods to document subtle changes in multimer distribution in plasma; (*ii*) difficulties in establishing such a relationship without carefully controlled work because of variability in the multimer patterns between individuals; (*iii*) selective consumption of larger multimers in plasma; or (*iv*) the fact that 10% ADAMTS13 activity is sufficient to proteolytically process unusually large VWF as seen in patients receiving plasma for the treatment of ADAMTS13 deficiency (21). Some of these points may result in the compensation of the bleeding tendency in severe hemophilia A and offer a potential explanation for the heterogeneous bleeding tendency in these patients.

In summary, we conclude that FVIII functions as a cofactor in accelerating processing of VWF by ADAMTS13 under shear stress. This rate-enhancing effect depends on the ability of FVIII to bind to VWF with high affinity. We speculate that the selective action of ADAMTS13 on larger VWF multimers likely arises from the probability of encountering more FVIII molecules bound to the larger multimeric species at physiological concentrations of FVIII and VWF.

Methods

Preparation of Recombinant and Native Proteins. Recombinant human full-length FVIII, obtained as a kind gift from Lisa Regan (Bayer), was repurified to remove serum albumin by cation exchange chromatography (22), exchanged into 20 mM Hepes, 0.15 M NaCl, and 5 mM $CaCl_2$ (pH 7.5), and stored at −80°C. A B-domainless derivative of FVIII (FVIII-SQ) was constructed by using the technique of splicing by overlap extension (23) using human FVIII cDNA (American Type Culture Collection) as a template. The product was subcloned into the pED expression vector obtained as a generous gift from Monique Davis (Wyeth) (24). FVIII-SQ lacks residues 744-1637 and has a 14-aa linker between the heavy (1–740; A1–A2 domains) and light (1649–2332; a3–A3–C1–C2) chains (Fig. 5*A*). FVIII-2RKR lacks the entire B domain and acidic region a3 (741–1689). A PACE/furin recognition site (RKRRKR) was inserted between the heavy chains (1–740) and the light chains (1690–2332) to facilitate intracellular proteolytic processing (Fig. 5*A*). Plasmids were transfected into baby hamster kidney (BHK) cells, and stable clones were established essentially as described (25). Recombinant FVIII derivatives were purified by using procedures described with minor modifications (25). rVWF was expressed in BHK cells overexpressing PACE/furin and purified from conditioned media by immunoaffinity chromatography using monoclonal antibody RU-8 as described (26). pVWF was purified from cryoprecipitate as described (27). Recombinant ADAMTS13 containing a V5-His tag at the C terminus was expressed in HEK293 cells and purified according to published procedures (18). Thrombin was prepared from prothrombin and purified as described (28). Protein purity was assessed by SDS/PAGE under reducing conditions, followed by staining with Coomassie blue. Protein concentrations were determined by using the following molecular weights and extinction coefficients (E_{280}, 1 mg/ml): FVIII, 264,700, 1.22 calculated from amino acid composition (29); FVIII-SQ and FVIII-2RKR, 160,000, 1.6 (30); ADAMTS13, 195,000, 0.68 (18); VWF, 250,000, 1.0 (6).

Cleavage of VWF by ADAMTS13 Under Shear Stress. Purified pVWF or rVWF (37.5 μg/ml or 150 nM) was incubated at 25°C for 3 min or the indicated times with 50 nM recombinant ADAMTS13 in the absence or presence of FVIII, FVIII-SQ, FVIII-2RKR, or FVIIIa (0–40 nM) in 20 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.5 mg/ml BSA (pH 7.5) under constant vortexing at 2,500 rpm. Experiments were performed in 0.2-ml thin-walled PCR tubes (Fisher Scientific, Newark, DE) with a final reaction volume of 20 μl as described previously (18). The reaction was quenched at various times by adding an equal volume of 125 mM Tris, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, and 0.01% (wt/vol) bromophenol blue (pH 6.8), followed by heating at 100°C for 5 min. Samples were run on a 5% Tris-glycine SDS/PAGE gel and then transferred to nitrocellulose. The membrane was blocked with 1% (wt/vol) casein in 20 mM Tris·HCl, 0.15 M NaCl, and 0.05% (vol/vol) Tween 20 (TBSTc) and then incubated with rabbit anti-VWF IgG (DAKO) in TBSTc for 2 h or overnight at 25°C. After washing with TBST, the blot was incubated for 1 h with IRDye 800CW-labeled goat anti-rabbit IgG (LI-COR Bioscience) in TBSTc. An Odyssey Infrared Imaging System (LI-COR Bioscience) was used to quantify the fluorescent signal of the cleavage product (M_r = 350,000).

VWF Multimer Analysis. After digestion under various conditions, samples were denatured by heating at 60°C for 20 min in 70 mM Tris, 2.4% (wt/vol) SDS, 0.67 M urea, and 4 mM EDTA (pH 6.5) and fractionated in a gel containing 1.5% (wt/vol) SeaKem HGT agarose (Cambrex). Protein was transferred onto polyvinylidene fluoride membranes (Millipore) by capillary diffusion. Blots were processed for immunodetection as described above.

Cleavage of VWF by ADAMTS13 Under Denaturing Conditions. Purified pVWF (3.0 μM) was predenatured with 1.5 M guanidine at 37°C for 2 h. After a 1:10 dilution, VWF was incubated with 12.5 nM recombinant ADAMTS13 at 37°C for 1 h in the absence or presence of FVIII (0–40 nM) in assay buffer. The 350K cleavage product was detected by Western blot analysis as described above.

Binding of FVIII Derivatives to Solid-Phase VWF. Wells of a microtiter plate were coated with VWF (10 μg/ml) and blocked with 1% casein in PBS (pH 7.4). FVIII-SQ and FVIII-2RKR (0–20 nM) in PBS with 0.1% casein were incubated with immobilized VWF for 1 h. After washing with PBS, bound FVIII-SQ or FVIII-2RKR was detected in an ELISA format by using a monoclonal anti-FVIII antibody (ESH-8) against the C2 domain of FVIII (kindly provided by Weidong Xiao) and peroxidase-conjugated goat anti-mouse IgG (DAKO, Carpinteria, CA).

Cleavage of FRETS-VWF73. ADAMTS13 (12.5 nM) and FVIII (0–40 nM) were preincubated for 5 min at room temperature, and FRETS-VWF73 substrate (2

μM) in 5 mM Bis-Tris, 25 mM $CaCl_2$, and 0.005% Tween 20 (pH 6.0) was then added (31). The cleavage of FRETS-VWF73 was monitored by using λ_{EX} = 340 nm and λ_{EM} = 450 nm at 30°C with a Wallac 1420 VICTOR^3 fluorescent plate reader (PerkinElmer) to determine initial rates of cleavage.

Binding of FVIII to ADAMTS13. Recombinant ADAMTS13 was coupled to a carboxymethylated dextran plasmon resonance chip (≈2,000 response units; 2–10 ng/mm^2) by using methods described previously (18). Casein was immobilized in a similar way in the control channel, and both surfaces were blocked by using 1 M ethanolamine (pH 8.5). FVIII derivatives (0–40 nM) in 20 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.005% (vol/vol) Tween 20 (pH 7.5) were passed over the chip at a rate of 20 μl/min for 3 min, and sensograms were recorded in a BiaCore2000 instrument. After subtraction of nonspecific binding, binding curves were analyzed by fitting the data of maximal response units at equilibrium against the concentrations of FVIII derivatives.

ACKNOWLEDGMENTS. We thank Dr. Ping Zhang for assistance in preparing ADAMTS13, Hua Zhu (Children's Hospital of Philadelphia) for FVIII plasmids and cell lines, and Dr. Raffaella Toso (Children's Hospital of Philadelphia) for FVIII-SQ and FVIII-2RKR. We also thank Drs. Ann H. Rux and Bruce S. Sachais for their technique support for Biosensor experiments. This work was supported in part by National Institutes of Health Grants HL079027 (to X.L.Z.), HL-62523 (to S.K. and R.M.C.), and HL-47465 (to S.K.); the Bayer Hemophilia Awards Program (R.M.C.); and a Chinese Council Scholarship (to W.C.).

1. Tsai HM (1996) Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 87:4235–4244.
2. Moake JL, *et al.* (1982) Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 307:1432–1435.
3. Shim K, Anderson PJ, Tuley EA, Wiswall E, Sadler JE (2007) Platelet-VWF complexes are preferred substrates of ADAMTS13 under fluid shear stress. *Blood* 111:651–657.
4. Furlan M, Robles R, Lammle B (1996) Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 87:4223–4234.
5. Tsai HM, Lian EC (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 339:1585–1594.
6. Anderson PJ, Kokame K, Sadler JE (2006) Zinc and calcium ions cooperatively modulate ADAMTS13 activity. *J Biol Chem* 281:850–857.
7. Mann KG, Jenny RJ, Krishnaswamy S (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu Rev Biochem* 57:915–956.
8. Nishio K, Anderson PJ, Zheng XL, Sadler JE (2004) Binding of platelet glycoprotein Ibalpha to von Willebrand factor domain A1 stimulates the cleavage of the adjacent domain A2 by ADAMTS13. *Proc Natl Acad Sci USA* 101:10578–10583.
9. Lenting PJ, Van Mourik JA, Mertens K (1998) The life cycle of coagulation factor VIII in view of its structure and function. *Blood* 92:3983–3996.
10. Federici AB (2003) The factor VIII/von Willebrand factor complex: Basic and clinical issues. *Haematologica* 88:3–12.
11. Vlot AJ, Koppelman SJ, van den Berg MH, Bouma BN, Sixma JJ (1995) The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood* 85:3150–3157.
12. Lollar P, Parker CG (1987) Stoichiometry of the porcine factor VIII-von Willebrand factor association. *J Biol Chem* 262:17572–17576.
13. Lollar P, Hill-Eubanks DC, Parker CG (1988) Association of the factor VIII light chain with von Willebrand factor. *J Biol Chem* 263:10451–10455.
14. Hill-Eubanks DC, Lollar P (1990) von Willebrand factor is a cofactor for thrombin-catalyzed cleavage of the factor VIII light chain. *J Biol Chem* 265:17854–17858.
15. Lollar P, Knutson GJ, Fass DN (1985) Activation of porcine factor VIII:C by thrombin and factor Xa. *Biochemistry* 24:8056–8064.
16. Fay PJ, Smudzin TM (1992) Characterization of the interaction between the A2 subunit and A1/A3–C1-C2 dimer in human factor VIIIa. *J Biol Chem* 267:13246–13250.
17. van den Biggellaar M, Bierings R, Storm G, Voorberg J, Mertens K (2007) Requirements for cellular co-trafficking of factor VIII and von Willebrand factor to Weibel-Palade bodies. *J Thromb Haemost* 5:2235–2243.
18. Zhang P, Pan W, Rux AH, Sachais BS, Zheng XL (2007) The cooperative activity between the carboxyl-terminal TSP-1 repeats and the CUB domains of ADAMTS13 is crucial for recognition of von Willebrand factor under flow. *Blood* 110:1887–1894.
19. Grunewald M, *et al.* (2002) Absence of compensatory platelet activation in patients with severe haemophilia, but evidence for a platelet collagen-activation defect. *Platelets* 13:451–458.
20. Rock G, Adamkiewicz T, Blanchette V, Poon A, Sparling C (1996) Acquired von Willebrand factor deficiency during high-dose infusion of recombinant factor VIII. *Br J Haematol* 93:684–687.
21. Furlan M, Lammle B (1998) Deficiency of von Willebrand factor-cleaving protease in familial and acquired thrombotic thrombocytopenic purpura. *Baillieres Clin Haematol* 11:509–514.
22. Fay PJ, Haidaris PJ, Smudzin TM (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.
23. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* 77:61–68.
24. Kaufman RJ, Davies MV, Wasley LC, Michnick D (1991) Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. *Nucleic Acids Res* 19:4485–4490.
25. Toso R, Camire RM (2004) Removal of B-domain sequences from factor V rather than specific proteolysis underlies the mechanism by which cofactor function is realized. *J Biol Chem* 279:21643–21650.
26. Lankhof H, *et al.* (1999) Recombinant VWF type 2A mutants R834Q and R834W show a defect in mediating platelet adhesion to collagen, independent of enhanced sensitivity to a plasma protease. *Thromb Haemost* 81:976–983.
27. Zheng XL, Nishio K, Majerus EM, Sadler JE (2003) Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. *J Biol Chem* 278:30136–30141.
28. Lundblad RL, Kingdon HS, Mann KG (1976) Thrombin. *Methods Enzymol* 45:156–176.
29. Gill SC, von Hippel PH (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* 182:319–326.
30. Curtis JE, Helgerson SL, Parker ET, Lollar P (1994) Isolation and characterization of thrombin-activated human factor VIII. *J Biol Chem* 269:6246–6251.
31. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T (2005) FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 129:93–100.