ADAMTS13 Substrate Recognition of von Willebrand Factor A2 Domain

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ADAMTS13 controls the multimeric size of circulating von Willebrand factor (VWF) by cleaving the Tyr1605–Met1606 bond in the A2 domain. To examine substrate recognition, we expressed in bacteria and purified three A2 (VWF76–(1593–1668), VWF115–(1554–1668), VWFA2–(1473–1668)) and one A2-A3 (VWF115-A3–(1554–1874)) domain fragments. Using high pressure liquid chromatography analysis, the initial rates of VWF115 cleavage by ADAMTS13 at different substrate concentrations were determined, and from this the kinetic constants were derived (K_m 1.61 μM; k_cat 0.14 s⁻¹), from which the specificity constant k_cat/K_m was calculated, 8.70 × 10⁴ M⁻¹ s⁻¹. Similar values of the specificity constant were obtained for VWF76 and VWF115-A3. To identify residues important for recognition and proteolysis of VWF115, we introduced certain type 2A von Willebrand disease mutations by site-directed mutagenesis. Although most were cleaved normally, one (D1614G) was cleaved 8-fold slower. Mutagenesis of additional charged residues predicted to be in close proximity to Asp⁶¹⁴ on the surface of the A2 domain (R1583A, D1587A, D1614A, E1615A, K1617A, E1638A, E1640A) revealed up to 13-fold reduction in k_cat/K_m for D1587A, D1614A, E1615A, and K1617A mutants. When introduced into the intact VWFA2 domain, proteolysis of the D1587A, D1614A, and E1615A mutants was also slowed, particularly in the presence of urea. Surface plasmon resonance demonstrated appreciable reduction in binding affinity between ADAMTS13 and VWF115 mutants (K_D, up to ~1.3 μM), compared with VWF115 (K_D 20 nM). These results demonstrate an important role for Asp⁶¹⁴ and surrounding charged residues in the binding and cleavage of the VWFA2 domain by ADAMTS13.

Von Willebrand factor (VWF) is a large multimeric plasma glycoprotein that mediates tethering and adhesion of circulating platelets at sites of vascular injury (1). Following endothelial damage, plasma VWF binds to the exposed sub-endothelial collagen (2). Once immobilized, the shear forces of the flowing blood induce a conformational transition that critical residues in the vicinity of the cleavage site are required for optimum cleavage and form part of an ADAMTS13 docking site within the A2 domain (12–14). The latter are proximate or within the A2 domain and manifest enhanced ADAMTS13-dependent VWF proteolysis. This is thought to be because of structural changes that lead to constitutive/enhanced exposure of the A2 domain cleavage site (15).

Because of the uncertainty surrounding A2 domain recognition mechanisms by ADAMTS13, we have prepared both wild-type and variant A2 domain fragments that span the Tyr1605–Met1606 cleavage site. For the first time, the cleavage of these by ADAMTS13 has been evaluated kinetically under normal physiological ionic conditions. We show that critical residues in the vicinity of the cleavage site are required for optimum cleavage and form part of an ADAMTS13 docking site within the A2 domain.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant ADAMTS13 and VWF Fragments—Recombinant human ADAMTS13 with a C-terminal Myc/His₆ tag was expressed and purified as previously described (16). For
surface plasmon resonance (SPR) and kinetic analysis experiments, an additional gel filtration step, using a Sephadex 26/10 column (Amersham Biosciences), was employed to obtain higher purity ADAMTS13.

The coding regions for different VWF A domain fragments (VWF76, Ser1593-Arg1668; VWF115, Glu 1554-Arg1668; VWF115-A3, Glu 1554-Glu1874; VWFA2, Met 1473-Arg1668) were PCR amplified from the human VWF cDNA using Pfx high fidelity DNA polymerase (Invitrogen) according to the manufacturer’s instructions. Fragments were cloned into the bacterial expression vector pET100/D-TOPO (Invitrogen), which fuses a His6 and Xpress epitope tag to the N terminus of cloned sequences. As the VWF A3 domain contains two disulfide bonds, the adjacent paired cysteines at positions 1669 and 1670 (**) were mutated to alanine using the QuikChange XL kit (Stratagene). This approach improved yields of VWF115-A3 and ensured that the conserved A domain disulfide bond between Cys1686-Cys1872 was correctly paired. Certain type 2A VWD mutations (R1597W, R1597Q, D1614G, I1628T, G1629R, E1638K) and specific charged residue substitutions (R1583A, D1587A, D1614A, E1615A, K1617A, E1638A, E1640A) were introduced into VWF115 and VWFA2 sequences using the QuikChange XL kit (Stratagene). All vectors were verified by sequencing.

VWF76, VWF115, and VWFA2 constructs were expressed in Rosetta Escherichia coli (Novagen, Nottingham, UK). VWF115-A3 was expressed in Rosetta-Gami E. coli, which allows cytoplasmic disulfide bond formation. Transformed bacteria were grown in 2X yeast tryptone cultures at 37 °C with shaking. Once in log-phase growth,

VWF A2 Domain and ADAMTS13
recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside for 16 h. Bacteria were harvested, and soluble (cytoplasmic) or insoluble (inclusion body) fractions were prepared using BugBuster reagent (Novagen). Recombinant His-tagged proteins were purified on an ÄKTA FPLC (Amersham Biosciences) using Ni²⁺-HiTrap-chelating columns (Amersham Biosciences). VWF76 was purified from the soluble fraction according to the manufacturer’s instructions and eluted with 500 mM imidazole. VWF115-A3, VWF115, and VWFFA2 inclusion bodies were solubilized in 8 M urea/20 mM Tris-HCl (pH 7.8)/50 mM imidazole and bound to the Ni²⁺-chelating column. Protein refolding was achieved using an 8–0 M urea linear gradient (1 ml/min for 40 min). Refolded, soluble material was eluted with 500 mM imidazole. Misfolded, precipitated protein was stripped from the column with 6 M guanidine/0.5 M EDTA. All recombinant proteins (eluted and stripped) were dialyzed into 20 mM Tris (pH 7.8) overnight at 4 °C. For VWFFA2 and VWF115-A3, the stripped material was refolded by this process. Thereafter, soluble and insoluble protein fragments were separated by centrifugation. The purity of each recombinant protein was assessed by SDS-PAGE and Coomassie staining and quantified using a BCA total protein assay kit (Perbio, Cramlington, UK).

Cleaveage of VWF Fragments by ADAMTS13—For time course reactions, recombinant ADAMTS13 (final reaction concentration 5–16.5 nM) was preincubated without substrate at 37 °C for 30 min in reaction buffer (final reaction concentrations 20 mM Tris, pH 7.8/150 mM NaCl/5 mM CaCl₂). In preliminary experiments, VWF76, VWF115, VWF2A, or VWF115-A3 were added as final concentrations of 4–8 μM to start each reaction. For analyses of the intact A2 domain, reactions were performed in the presence and absence of 1 M urea. At different time points, 65-μl sub-samples were removed and stopped with EDTA. 15 μl were analyzed by SDS-PAGE (16.5% Tris-Tricine gel; Invitrogen) and Coomassie staining. The remaining 50 μl were analyzed by HPLC. Samples were loaded on a BioBasic C4 column (Thermo Electron, Roncun, UK), and the full-length and cleaved peptides were eluted/resolved using a specific acetonitrile gradient (20–55% for VWF76, 25–45% for VWF115, 25–60% for VWF2A, 20–65% for VWF115-A3). Mass spectrometry or N-terminal sequencing (Alta Bioscience, Edgbaston, UK) was performed to identify the polypeptide in each eluted peak (Protein Data Bank code 1atz_A), according to the method outlined by Sutherland et al. (17). Models were manipulated using Protein Explorer (www.molvis.sdc.edu/protxpl/frntdoor.html).

RESULTS

Purification of Recombinant VWF Fragments—Different human VWF A2 domain substrate fragments (Fig. 1A) were expressed in, and purified from, bacteria. VWF76 was purified from the soluble, cytoplasmic fraction by Ni²⁺-chelating column affinity chromatography (Fig. 1B). SDS-PAGE and Coomassie staining demonstrated a purified product of 12.5 kDa. VWF115, VWF2A, and VWF115-A3 were purified from inclusion body preparations. Refolding of these proteins was achieved using an 8–0 M urea linear gradient during purification. This technique produced high yields of soluble VWF115 and VWF2A of the correct predicted size (16.9 and 26.9 kDa, respectively) that were essentially pure (Fig. 1, C and D). VWF115-A3 was initially purified and refolded similarly. However, after elution a relatively low yield of soluble material was purified (Fig. 1E, lane 1). The precipitated protein was stripped from the column and refolded by dialysis, yielding higher amounts of soluble material (lane 2) than the on-column refolded preparation. The eluted material and the refolded/solubilized stripped fragment obtained after dialysis contained a product of 38.6 kDa (lanes INR and 2NR). The presence of the disulfide bond between Cys¹⁵⁸⁶ and Cys¹⁸⁷² was confirmed by the band shift upon reduction of this material (lanes 1R and 2R). The insoluble material after dialysis contained VWF115-A3 polymers arising through intermolecular disulfide bond formation (lane 3) and was discarded.

Proteolysis of VWF76, VWF115, and VWF115-A3—To first visualize the capability of ADAMTS13 to proteolyze each of the recombinant A2 domain fragment substrates, recombinant ADAMTS13 was incubated at 37 °C with either VWF76, VWF115, or VWF115-A3 in the presence of physiological concentrations of Ca²⁺ and NaCl. Sub-samples were stopped between 0 and 6 h and analyzed by SDS-PAGE and Coomassie staining to give a qualitative assessment of each reaction (Fig. 2, A–C). In each case, only the full-length substrate band was visible at 0 h. Thereafter, the intensity of this band diminished and two smaller cleavage

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product bands appeared. In each case, these bands corresponded to the C-terminal fragment, in each case starting at Met1606. Visually, VWF76, VWF115, and VWF115-A3 were, as indicated by the Coomassie staining, very similar (not shown).

Kinetic Analysis of VWF76, VWF115, VWF115-A3, and VWF115 Charged Mutant Proteolysis—As a qualitative assessment of the relative ability of ADAMTS13 to proteolyze each of the charged residue mutants compared with the wild-type VWF76, VWF115, and VWF115-A3 substrates, we analyzed cleavage reactions by SDS-PAGE and Coomassie staining (Fig. 4A). From these gels, it seemed as if all of the mutants exhibited reduced rates of proteolysis. However, the concentrations of substrates needed to facilitate visualization by gel staining were high. To perform kinetic analysis of proteolysis for different substrates, it was necessary to monitor cleavage using substrate concentrations (250–500 nM). Repeated time course experiments were performed using low substrate concentrations (100 nM–50 μM). For the ADAMTS13-dependent proteolysis of VWF115 (n = 4), we derived a \( k_{cat} \) of 0.14 ± 0.06 s\(^{-1}\). The \( K_m \) for this reaction was determined to be 1.61 ± 0.47 μM, from which a value for the specificity constant (catalytic efficiency, \( k_{cat}/K_m \)) of 8.70 × 10\(^4\) M\(^{-1}\) s\(^{-1}\) could be derived.

For separate determination of the catalytic efficiency of VWF115, repeated time course experiments were performed using low substrate concentrations (250–500 nM). Repeated time course experiments (n = 8) gave a mean value of 7.83 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) for \( k_{cat}/K_m \) (Table 1). Confirmation of the pseudo-first order conditions was obtained by deriving the same \( k_{cat}/K_m \) value for VWF115 proteolysis at both 250 and 500 nM. Determination of \( k_{cat}/K_m \) for VWF76 and VWF115-A3 gave values of 6.39 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) and 6.70 × 10\(^3\) M\(^{-1}\) s\(^{-1}\), respectively (Table 1). These kinetic data confirmed that the VWF3 domain does not significantly contribute to the rate of A2 domain proteolysis, despite containing an ADAMTS13 binding site (18).

The contribution of each selected charged amino acid to VWF proteolysis was assessed by determination of the catalytic efficiency of all the mutant VWF115 substrates at 500 nM (Fig. 4C and Table 1). The rate
of proteolysis of the R1583A mutant was not significantly different from that of VWF115, suggesting that this residue is not important for cleavage. Mutation of Glu1638 and Glu1640 induced a mild (2–3-fold) reduction in catalytic efficiency. The most affected (12-fold reduction) single mutant was D1587A, the $k_{cat}/K_m$ of which was $0.64 \pm 0.10 \times 10^4$ M$^{-1}$ s$^{-1}$. The $k_{cat}/K_m$ for K1617A, D1614A, and E1615A, which are predicted to lie on the same surface-exposed loop, were 1.90, 1.36, and 1.13 $\times 10^4$ M$^{-1}$ s$^{-1}$, respectively. These results implied that this loop might play an important role in the cleavage reaction. Consequently, we generated a triple mutant DEK1614/16/5AAA) containing all 3 residues substituted for alanine. Whereas individually these mutations caused a 4–7-fold reduction in $k_{cat}/K_m$, in combination the catalytic efficiency was >13-fold less than wild-type VWF115, implying that the amino acids in this charged loop may act in synergy.

**Influence of D1587A, D1614A, and E1615A upon VWF1Acleavage.** To test whether the effects of the above mutations were maintained in a fully folded A2 domain, we introduced the most affected single charged mutants into the whole A2 domain. A variety of VWF A2 domain mutations appear to alter the conformation of the domain, such that the scissile bond is more readily exposed and thus more accessible for ADAMTS13 cleavage under normal physiological ionic conditions. Therefore, this approach also served to ascertain whether the most affected charged residue mutants (D1587A, D1614A, E1615A) induced gross structural changes. Despite using increased concentrations of ADAMTS13 (16.5 nM) in the absence of a denaturant, the intact wild-type A2 domain was proteolyzed very slowly, as visualized by gel staining (Fig. 5A), when compared with the A2 domain fragments VWF115, VWF76, and VWF115-A3. When samples containing 500 nM VWFA2
were analyzed by HPLC (Fig. 5C), it was clear that the cleavage reaction did not proceed toward completion (~28% after 5 h), as previously reported (13). The D1587A, D1614A, and E1615A VWFA2 mutants were proteolyzed either at a similar or slower rate (Fig. 5, A and C), suggesting that these mutations do not alter the A2 domain structure markedly. Under denaturing conditions (1 M urea), the wild-type VWFA2 was proteolyzed faster, although it still did not reach completion (~50% after 5 h) (Fig. 5, B and D). Although all the mutations were cleaved at a higher rate under denaturing conditions, they were cleaved appreciably slower than VWFA2, corroborating the direct influence of these mutations upon VWFA15 proteolysis.

Confirmation of ADAMTS13 Binding Site Residues in VWFA2 Domain—We hypothesized that the most likely cause of the reduced catalytic efficiency of the affected VWFA15 charged mutants was impaired binding to ADAMTS13. To measure the influence of each mutation upon the interaction of VWFA15 with ADAMTS13, we performed SPR analysis. Monoclonal antibody-mediated orientation-specific coupling of either VWFA15 or ADAMTS13 to the sensor chip was not possible due to the relatively fast off-rates of these molecules with the available antibodies to each ligand (not shown). Consequently, we covalently bound either VWFA15 or ADAMTS13 to the sensor chip by amine coupling. With VWFA15 bound to the chip and ADAMTS13
injected over the sensor, we monitored a concentration-dependent binding response indicative of a high affinity interaction ($K_D \approx 20$ nM) for this interaction (Fig. 6, inset). Determination of both the association and dissociation rates for the ADAMTS13-VWF115 interaction ($n = 3$) demonstrated a slow off-rate ($1.72 \times 10^{-3}$ s$^{-1}$), which would appear to be the major determinant for the high affinity binding. A second approach, in which ADAMTS13 was coupled to the sensor chip and varying concentrations of VWF115 injected, gave a similar high affinity binding component ($K_D \approx 15$ nM, not shown). These data demonstrate that the affinity of ADAMTS13 for VWF115 is significantly higher than is suggested by the $K_m$ for VWF115 proteolysis.

SPR analysis of VWF115 mutants (D1587A, D1614A, E1615A, and DEK1614/5/7AAA) using both binding approaches revealed a markedly reduced affinity of ADAMTS13 for each of the mutants when compared with wild-type VWF115 (Fig. 6). Because of the low binding response measured, an accurate $K_D$ could not be derived from the VWF115 mutant data. Despite the poor fitting the estimate for the $K_D$ for the DEK1614/5/7AAA mutant was $1.3 \times 10^6$ M. It was clear that for each variant the binding with ADAMTS13 was appreciably compromised and thus the likely cause of their reduced specificity constants.

**DISCUSSION**

ADAMTS13 cleaves a single bond within the VWF A2 domain (9).
Under static or low shear conditions, proteolysis is slow because of the globular conformation adopted by multimeric VWF. Moreover, the complex folding of the A2 and surrounding domains further conceals the scissile bond (10). The isolated intact A2 domain is also not readily proteolyzed by ADAMTS13 (19). In the absence of a denaturant, the intact domain (VWF residues Met1473-Arg1668) adopts a "non-permissive" structure that restricts access of ADAMTS13 (13). This is potentially explained by a VWF A2 domain model that suggests that the Tyr1605–Met1606 bond lies buried within the center of the folded domain (17) and is thus not normally solvent exposed. However, incomplete VWF A2 domain fragments that span the ADAMTS13 cleavage site (e.g. VWF73 Asp1595-Arg1668 and VWF115 Glu1554-Arg1668) have been shown, here and elsewhere (12, 20, 21), to be readily and specifically proteolyzed by ADAMTS13 in the presence of denaturant.

**TABLE 1**
Kinetic analysis of different VWF substrate proteolysis by ADAMTS13.

ADAMTS13 was incubated with 500 nM substrate under physiological ionic conditions at 37 °C. Sub-samples from each time course reaction were taken (0–6 h) and the extent of proteolysis measured by HPLC. The catalytic efficiency ($k_{cat}/K_m$) of proteolysis of each substrate was derived using Enzfitter software. The specificity constants for each VWF substrate/VWF115 variant is given (± S.E.). For VWF115, VWF115-A3, and VWF76, $n = 8$. For the least affected VWF115 variants (R1583A, D1640A, E1638A, and K1617) and the triple mutant DEK1614/5/7AAA, $n = 1$. For the most affected single mutants (D1614A, E1615E, and D1587), $n = 3$. Numbering as in Fig. 4.

<table>
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<tr>
<th>VWF substrate</th>
<th>$k_{cat}/K_m$ ($\times 10^6$ nM$^{-1}$s$^{-1}$)</th>
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<tr>
<td>VWF115</td>
<td>7.83 ± 1.18</td>
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<tr>
<td>VWF115-A3</td>
<td>6.70 ± 2.78</td>
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<tr>
<td>VWF76</td>
<td>6.39 ± 1.18</td>
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<tr>
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<tr>
<td>D1640A (7)</td>
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<tr>
<td>E1638A (6)</td>
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<tr>
<td>K1617A (5)</td>
<td>1.90</td>
</tr>
<tr>
<td>D1614A (3)</td>
<td>1.36 ± 0.26</td>
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<tr>
<td>E1615 (4)</td>
<td>1.13 ± 0.30</td>
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<tr>
<td>D1587A (2)</td>
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<tr>
<td>D1614A/E1615A/K1617A (8)</td>
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**FIGURE 5.** VWF A2 and VWF A2 charged mutant proteolysis. A, 6 μM VWF A2 (1) or VWF A2 carrying D1587A (2), D1614A (3), or E1615A (4) was incubated with 16.5 nM ADAMTS13 in the absence of denaturant and analyzed by SDS-PAGE and Coomassie staining. Samples were incubated for 0, 1, and 3 h as indicated. B, 6 μM VWF A2 (5) or VWF A2 carrying D1587A (6), D1614A (7), or E1615A (8) mutations was incubated with ADAMTS13 as in panel A in the presence of 1 μM urea. Samples were analyzed as in panel A. C, graph of HPLC analysis of time course reaction samples 1–4 (panel A) set up in parallel reactions containing 500 nM of each substrate. D, graph of HPLC analysis of time course reaction samples 5–8 (panel B) set up in parallel reactions containing 500 nM of each substrate.
cleaved at the Tyr\textsuperscript{1605}–Met\textsuperscript{1606} site under normal physiological ionic conditions, suggesting that these fragments not only contain important recognition sequences for specific ADAMTS13-dependent proteolysis but also that their structure presents the cleavage site in a permissive conformation, more akin to unreacted/denatured VWF (12). In the present study, we have expressed and purified a number of related VWF A2 and A2/A3 domain fragments to delineate those regions important for A2 domain proteolysis. Previous studies have employed SDS-PAGE and/or Western blotting to monitor proteolysis, the latter to achieve clear separation of the cleaved from the uncleaved substrate, three of the mutations (D1587A, D1614A, and E1615A) were appreciably impaired. These results with both VWF115 and A2 substrates suggested that specificity is appreciably influenced by a charged patch on the surface of the A2 domain.

Binding of ADAMTS13 to multimeric VWF and A2 domain fragment substrates has been reported to be of high affinity (23). Physiologically, ADAMTS13 seems to circulate in complex with VWF. However, the binding of ADAMTS13 to VWF need not necessarily be accompanied by proteolysis. Although Ca\textsuperscript{2+} ions are required for efficient proteolysis (10), they are not necessary for high affinity binding (21, 23). A recent report of binding of ADAMTS13 to VWF73 in the absence of Ca\textsuperscript{2+} ions using a microtiter plate-based immunosassay for ADAMTS13 suggested a \( K_{D} \) of 4.6 nM (21). Because of uncertainties surrounding the use of antibody detection and equilibrium perturbation in the detection step of binding in such assays, we used the alternative approach of SPR. Coupling VWF115 directly to the sensor chip and injecting different concentrations of ADAMTS13 yielded a \( K_{D} \) of ~20 nM. This high affinity interaction appeared to be conferred by a slow dissociation rate \( (k_{d}) \). Confirmation of tight binding was obtained by covalently immobilizing ADAMTS13 and then injecting VWF115 over the sensor chip. In this case, a \( K_{D} \) of 15 nM was obtained. However, there was also some evidence for heterogeneity of binding, and a lower affinity component was also apparent using this second approach. Importantly though, both SPR approaches demonstrated substantial loss (>10-fold) in binding affinity of ADAMTS13 to the mutant VWF115 fragments D1587A, D1614A, and E1615A and the triple mutant DEK1614/5/7AAA irrespective of whether SPR was performed with these fragments as analyte or ligand. This provides direct evidence that these residues form part of a docking site for ADAMTS13 on the VWF115 domain.

The present investigation has for the first time identified critical residues within the VWF A2 domain that are important for both binding to ADAMTS13 and for proteolysis at the Tyr\textsuperscript{1605}–Met\textsuperscript{1606} bond. Given its specificity, proteolysis is unlikely to be determined solely by interaction with a limited number of residues surrounding the cleavage site. Previ-
ous studies have shown that although VWF73 is cleaved by ADAMTS13, a 9-residue deletion at the C terminus of this fragment completely abolished cleavage (12). It remains uncertain precisely where these 9 amino acids lie in the fully folded intact A2 domain or in unraveled multimeric VWF in relation to the charged patch/residues identified in this study. Despite this, and the inability of a peptide that spans this region to inhibit proteolysis, it remains possible that residues within this 9-amino acid sequence contribute directly to high affinity binding to the protease (12). Structures remote from the cleavage site are also known to influence proteolysis. The A1 domain of VWF was recently demonstrated to inhibit access of ADAMTS13 to the A2 domain cleavage site (13). This inhibition was removed in the presence of GpIbα, the principal A1 domain ligand, suggesting that under physiological conditions platelet binding may enhance VWF proteolysis either by increasing scissile bond access or by revealing an ADAMTS13 binding site.

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