

RECOMBINANT CUB-1 DOMAIN POLYPEPTIDE INHIBITS THE CLEAVAGE OF ULVWF STRINGS BY ADAMTS-13 UNDER FLOW CONDITIONS†

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ABSTRACT

The metalloprotease ADAMTS-13 converts the hyper-reactive unusually large (UL) forms of von Willebrand factor (VWF) that are newly released from endothelial cells into less active plasma forms by cleaving a peptide bond in the VWF A2 domain. Familial or acquired deficiency of this metalloprotease leads to the thrombotic disorder, thrombotic thrombocytopenic purpura (TTP). ADAMTS-13 belongs to the ADAMTS metalloprotease family, but, unlike other members, it also contains two C-terminal CUB domains (Complement component C1r/C1s, Uegf, and Bone morphogenic protein 1). Mutations in the CUB region have been found in congenital TTP, but deletion of the region did not impair enzyme activity in conventional *in vitro* assays. We investigated the functions of the CUB domain in ADAMTS-13 activity under flow conditions. We found that recombinant CUB-1 and CUB-1+2 polypeptides and synthetic peptides derived from the CUB-1 partially blocked the cleavage of ULVWF by ADAMTS-13 on the surface of endothelial cells under flow. The polypeptide bound immobilized and soluble forms of ULVWF, and blocked the adhesion of ADAMTS-13-coated beads to immobilized ULVWF under flow. These results suggest that the CUB-1 domain may serve as the docking site for ADAMTS-13 to bind ULVWF under flow, a critical step to initiate ULVWF proteolysis.

INTRODUCTION

In response to stimulation, vascular endothelial cells release unusually large (UL) and hyperactive von Willebrand factor (VWF) multimers^{1,2} that spontaneously bind the platelet GP Ib-IX-V complex in the absence of any modulators or high fluid shear stress.^{3,4} Because of this hyperactivity, ULVWF multimers released into the bloodstream spontaneously aggregate platelets and are responsible for the systemic microvascular thrombosis seen in patients with thrombotic thrombocytopenic purpura (TTP). To prevent such disastrous consequences, ULVWF multimers undergo limited proteolysis immediately upon release from endothelial cells by the zinc- and calcium-dependent metalloprotease ADAMTS-13 (A Disintegrin and Metalloprotease with ThromboSpondin motif).⁵⁻⁷ ADAMTS-13 cleaves VWF at a single peptide bond between Y842 and M843 in the VWF A2 domain, generating 176-kDa and 140-kDa fragments detectable on reducing SDS-PAGE gels.⁸⁻¹⁰ ADAMTS-13 deficiency has been demonstrated in patients with TTP,¹¹ caused either by mutations in the ADAMTS13 gene^{6,12-15} or by acquired antibody inhibitors of the metalloprotease.¹⁶⁻¹⁹

ADAMTS-13 has a domain structure similar to that of other family members, with the exception that it contains unique domains at its C-terminus, known as CUB domains.⁵ “CUB” is an acronym for the first three proteins described that contain this domain: complement components C1r/C1s, Uegf (sea urchin fibropellins), and bone morphogenic protein 1 (Bmp1). CUB domains have been found in functionally diverse proteins from prokaryotes to humans and are known to function primarily in protein–protein interactions.²⁰⁻²² Two individual mutations within the CUB domains of ADAMTS-13 have been identified in association with congenital TTP,^{6,13} suggesting that this domain is critical for the biosynthesis or activity of the metalloprotease. A potential functional role for the ADAMTS-13 CUB domains is also

suggested by a study showing that 64% of plasma samples from patients with acquired TTP contain antibodies against the CUB domains (all of them also contain antibodies against the Cys-rich and Spacer domains).²³ Nevertheless, the precise roles of the CUB domains in ADAMTS-13 cleavage of ULVWF remain unknown, particularly in the conditions found in flowing blood.

Using a parallel-plate flow chamber system, we previously showed that in the absence of ADAMTS-13, ULVWF multimers secreted from histamine-stimulated endothelial cells are anchored to the cell surface to form extraordinary long string-like structures that spontaneously bind platelets.^{4,24} Perfusion of plasma or recombinant ADAMTS-13 over the endothelial cells rapidly cleaves these ULVWF strings under physiological flow, suggesting that the cleavage of newly released ULVWF *in vivo* occurs predominantly on the endothelial surface. These anchored ULVWF strings containing adherent platelets are exposed to tensile stress applied by the flowing blood and are stretched to expose sites for ADAMTS-13 docking (VWF A1 and A3 domains) and cleavage (the VWF A2 domain).²⁵ It is still unknown, however, which domains or sequences within ADAMTS-13 attach the enzyme from the blood to the relatively stationary ULVWF strings. We hypothesize that it is the CUB domains, alone or in combination with other domains, that carry out this docking function. Here, we examined this hypothesis and found that the CUB domains, in particular CUB-1, bind ULVWF under both static conditions and under flow.

EXPERIMENTAL PROCEDURES

Platelet and plasma preparations

Freshly drawn blood from 20 healthy donors (12 females and 8 males of ages from 24-46) was used as the source of plasma and platelets under a protocol approved by the Institutional Review Board of the Baylor College of Medicine. All donors signed informed consent before blood was drawn. Washed platelets were obtained from blood in 10% acid-citrate dextrose buffer (ACD, 85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid) by centrifuging the blood first at $150 \times g$ for 15 min at 24°C to obtain platelet-rich plasma (PRP), and then centrifuging the PRP at $900 \times g$ for 10 min to collect platelets. The platelets were washed once with a CGS buffer (13 mM sodium citrate, 30 mM glucose and 120 mM sodium chloride, pH 7.0) and resuspended in Ca^{++} and Mg^{++} -free Tyrode's buffer (138 mM sodium chloride, 5.5 mM glucose, 12 mM sodium bicarbonate, 2.9 mM potassium chloride, and 0.36 mM dibasic sodium phosphate, pH 7.4).

As the source of ADAMTS-13, plasma was obtained from blood anticoagulated with D-Phe-Pro-Arg-chloromethylketone, HCl (PPACK, 75 μM final concentration, Calbiochem, La Jolla, CA).

Expression of recombinant ADAMTS-13 and isolated CUB domains

ADAMTS-13 cDNA⁷ was subcloned into the mammalian expression vector pSectag (Invitrogen) that carries the C-terminal His- and Myc-tag for purification and detection. The cDNA was introduced to HeLa cells, which contains endogenous furin to cleave propeptide and release the mature metalloprotease,^{26,27} by liposome-mediated DNA transfer (Lipofectamine TM 2000, Invitrogen). Transfected cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$

streptomycin, and 5 mM L-glutamine at 37°C with 95% air and 5% CO₂. Cells expressing the recombinant ADAMTS-13 (rADAMTS-13) were first selected in medium containing hygromycin-B (400 µg/ml) and cells expressing high levels of rADAMTS-13 was then selected by single cell cloning. Recombinant ADAMTS-13 was collected from the supernatant of confluent cells grown in serum-free medium (Opti-Pro SFM, Invitrogen) and purified through the C-terminal His-tag using a Y-perTM 6xHis Fusion Protein Purification Kit (Pierce Chemicals, Rockford, IL).

To express the recombinant CUB domains, DNA fragments encoding CUB-1 (A1191 to E1298), CUB-2 (C1299 to T1427), and CUB-1+2 (A1191 to T1427) were amplified from the ADAMTS-13 cDNA by polymerase chain reaction. To preserve structural integrity and remove the vector sequence (there are more than 30 nucleotides between the vector-derived signal cleavage site and the start of CUB-1 sequence), we have included a furin-cleavage site sequence RQRR to the N-terminus of each fragment. When the constructs are expressed in Hela cells, which express furin, the CUB-1 polypeptide can be generated without the foreign sequence. The CUB-1 and CUB-1+2 fragments were amplified using the same forward primer (ATGCAAGCTTCAGGCAGAGGAGGGCCTGTCAGGCAG) and the reverse primers ATGCCTCGAGCTTCTCTGTAGAAGGTTTCAGG and ATGCCTCGAGCGGTTCCCTTCCCTTCC, respectively. The CUB-2 was amplified with forward ATGCAAGCTTCAGGCAGAGGAGGGAATGTGACATGCAGCTC and reverse ATGCCTCGAGCGGTTCCCTTCCCTTCC primers. The amplified fragments were digested with Hind III and Xho 1, subcloned into the mammalian expression vector pSecTag2, and expressed in Hela cells as described for the wild-type rADAMTS-13. The recombinant CUB

polypeptides were again purified using a Y-per™ 6xHis Fusion Protein Purification Kit (Pierce Chemicals).

Synthetic CUB peptides

Five peptides derived from overlapping sequences of the CUB-1 domain (Table 1) were made by SigmaGenosys (Woodlands, TX). These peptides were chosen because their sequences are least homologous to that of CUB-2. Three control peptides were also synthesized: a scrambled peptide with the same amino acid composition as the peptide CUB-1A and two peptides from the catalytic domain of ADAMTS-13, which did not interfere with cleavage of ULVWF (data not shown). The peptides were dissolved in phosphate-buffered saline (PBS) to a stock concentration of 2 mM and stored at -20°C as small aliquots until use.

Endothelial culture

Human umbilical veins endothelial cells (HUVECs) were obtained under a protocol approved by the Institutional Review Board of the Baylor College of Medicine. The umbilical cords were washed with phosphate buffer and then incubated with 0.02% of collagenase (Invitrogen Life Technologies, Carlsbad, CA) for 30 min at room temperature. Endothelial cells were collected by centrifuging the elutes at $250 \times g$ for 10 min, then plated in a culture dish coated with 1% gelatin and grown in Medium 199 (Invitrogen Life Technologies) containing 20% heat-inactivated fetal bovine serum and 0.2 mM of L-glutamine.⁴ Before use, the cells were stimulated with 25 μM histamine (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature.

HUVECs were also used to produce ULVWF.²⁹⁻³¹ For this, the confluent HUVECs were first incubated with a serum-free M199 medium containing 5-10 $\mu\text{g/ml}$ of insulin, 5 $\mu\text{g/ml}$ of

transferrin, and 1% of glutamine for 48-72 hr and then treated with 100 μ M histamine for 30 min at 37°C to induce the release of ULVWF. The conditioned medium was collected and centrifuged at 350 \times g for 10 min to remove cell debris and the supernatant was used as the source of ULVWF. The multimeric composition of ULVWF from this preparation was evaluated by 1% SDS agarose gel electrophoresis and immunoblotting using a polyclonal VWF antibody (DakoCytomation, Carpinteria, CA).

Parallel-plate flow chamber

Culture dishes (35 mm) containing confluent monolayers of HUVECs served as the bottom of the chamber during assembly of the parallel-plate flow chamber. When necessary, HUVECs were stimulated with histamine before assembly of the chamber. The chamber was connected to a syringe pump to draw Tyrode's buffer containing washed platelets through the chamber at a flow rate of 0.2 ml/min, which generates a wall shear stress of 2.5 dyn/cm² for buffer or PRP (viscosity up to 1cp). ULVWF strings were quantitated by counting the numbers of strings in 20 continuous viewfields (400 \times) following a 2 min perfusion of platelet suspension.

Cleavage of ULVWF in the absence of flow

ADAMTS-13 activity was measured under static conditions using a method modified from that of Furlan *at al*^{8,32} with ULVWF multimers as the substrate. Briefly, normal plasma was diluted (1:5) with low ionic strength Tris-saline buffer and ADAMTS-13 was then activated with 1 mM BaCl₂ for 5 min. The plasma was then mixed with ULVWF, dialyzed against 1.5 M urea for 24 hr at 37°C, and then subjected to electrophoresis on a 1% non-reducing SDS agarose gel. The separated proteins were transferred to a PVDF membrane and VWF multimers detected by Western blot using a polyclonal anti-VWF antibody (DakoCytomation) and chemiluminescence.

We measured the amount of peptides recovered after a 24 hr dialysis and found to be 90% and 40% for the recombinant CUB-1 and synthetic CUB-1A peptide, respectively (data not shown).

ULVWF–CUB interaction

Interactions of ULVWF with wild-type ADAMTS-13, CUB domains, or a truncation mutant that lacks the sequence C-terminal to the Spacer domain (TSP-2)³³ were measured in the absence and presence of flow. For the static ELISA assay, the recombinant ADAMTS-13, CUB polypeptides (200 nM coating concentration), and the truncation mutant were immobilized onto the wells of microtiter plates (2 hr, room temperature). The coated plates were incubated with 5% BSA for 60 min to block non-specific sites, washed with PBS, and then incubated with ULVWF for additional 30 min at room temperature. After washing the wells with PBS, bound ULVWF was detected using a polyclonal VWF antibody (DakoCytomation) and chemiluminescence. The ADAMTS-13–ULVWF interaction was also examined in the presence of an antibody against the ADAMTS-13 CUB domains (10 µg/ml, Bethyl Laboratory, Houston, TX). This antibody was generated using, as immunogen, a synthetic peptide that contains the sequence of three peptides used in the blocking studies (peptide CUB-1A-C).

To examine the interaction of ADAMTS-13 with ULVWF under flow conditions, ADAMTS-13–coated polystyrene beads (0.5 µm in diameter, Polysciences, Inc., Warrington, PA) were perfused over immobilized ULVWF at a shear stress of 2.5 dyn/cm² and the adherent beads in 10 random review fields (400X) were counted after 5 min of perfusion.²⁵

Protein elution assay

ULVWF was incubated with either biotinylated synthetic CUB-1A peptide or a scrambled peptide of the same length and amino acid composition for 15 min at room temperature and the mixture was then passed through a gel filtration column with a molecular weight cut-off at 5000

(PD-10 column, Amersham Biosciences, Piscataway, NJ). Because of its very high molecular weight, ULVWF was normally eluted from the void volume at early fractions (fractions 3-7), whereas the peptides, which have a molecular weight of less than 1000, eluted at later fractions (after fraction 8). However, if the peptide formed a complex with ULVWF, it would appear in the ULVWF fraction. Fourteen fractions of 1 ml each were collected and 200 μ l of each fraction was applied to a nitrocellulose membrane by dot blot and the membrane was then probed for ULVWF and the CUB-1A peptides using a polyclonal anti-VWF antibody (DakoCytomation) and a monoclonal anti-biotin antibody (Pierce Biotechnology), respectively.

Statistical Analysis

All experimental data are presented as mean \pm SEM. The unpaired 2-tailed Student's *t* test was used for data analysis. Statistical significance was defined as having a *p* value of less than 0.05.

RESULTS

Recombinant CUB-1, but not CUB-2, blocked the cleavage of ULVWF strings by ADAMTS-13 under flow. We previously demonstrated that washed platelets in buffer perfused over stimulated HUVEC form string-like structures on newly released ULVWF multimers.⁴ No such structures formed if the platelets were suspended in plasma, a source of ADAMTS-13. Using this system, we examined the role of the ADAMTS-13 CUB domains in ULVWF cleavage by incubating PRP with recombinant CUB-1, CUB-2, or CUB-1+2 polypeptides before perfusion over histamine-stimulated HUVECs (2.5 dyn/cm² shear stress). The CUB-1 and CUB-1+2 polypeptides inhibited string cleavage by 72.4±11.6% and 39.8±8.4%, respectively, whereas the isolated CUB-2 did not (Figure 1A). Inhibition was dose-dependent, with maximal inhibition at 100 nM of the recombinant CUB-1 polypeptides (Figure 1B).

Consistent with this observation, we found that five synthetic peptides derived from the CUB-1 domain (CUB-1 peptides A through E, each at 200 µM) also partially inhibited ULVWF cleavage by ADAMTS-13, whereas cleavage was not inhibited by a scrambled peptide for CUB-1A or either of two peptides from the catalytic domain (Figure 2A). As with the recombinant CUB polypeptides, CUB-1A inhibition was partial, even up to a concentration of 1 mM (Figure 2B).

Recombinant CUB polypeptides did not block ULVWF cleavage under static conditions.

Compared to the results obtained under flow, ULVWF proteolysis was not inhibited by any of the recombinant CUB polypeptides or synthetic peptides in a static assay of ADAMTS-13 activity, using ULVWF as the substrate (Figure 3). This finding is consistent with previous studies that the ADAMTS-13 CUB domains are dispensable for cleaving plasma VWF under static conditions.^{26,34}

ULVWF interacts with CUB polypeptides under static conditions. The observation that recombinant CUB-1 blocked the cleavage of ULVWF under flow, but not under static conditions, suggests that the CUB-1 domain may dock ADAMTS-13 to ULVWF in flowing blood. We therefore examined the interaction of ULVWF with ADAMTS-13 or CUB polypeptides under static and flow conditions. Using ELISA, we found that ULVWF bound wild-type ADAMTS-13 and each of the three recombinant CUB polypeptides (Figure 4A). Of interest, ULVWF bound the CUB-2 polypeptide to a similar extent as it bound wild-type ADAMTS-13, CUB-1 and CUB-1+2, even though CUB-2 did not inhibit ULVWF cleavage under flow. One possible explanation for the observation is that the CUB-2–ULVWF interaction is too weak to withstand fluid shear stress. To test this possibility, we examined the adhesion of polystyrene beads coated with either wild-type ADAMTS-13 or with each of the CUB polypeptides to immobilized ULVWF under flow. Because the beads were unable to attach directly to ULVWF from the flowing buffer, we allowed the CUB-coated beads to settle onto the surface of immobilized ULVWF for 3 min before beginning the perfusion. Buffer was then perfused over the bead-covered surface for 2 min (shear stress 2.5 dyn/cm²) and the beads remaining adherent after this interval were counted. The hierarchy of bead binding was as follows: ADAMTS-13 > CUB-1 > CUB-1+2 > CUB-2. The difference between the binding of CUB-1 beads and CUB-2 beads was statistically significant (Student's t test, n = 10, p < 0.01, Figure 4B, black bars). We then determined the detachment of the adherent beads after 2 min perfusion under a high shear stress of 50 dyn/cm². The numbers of adherent beads coated with ADAMTS-13, CUB-1 or CUB-1+2 remained relatively unchanged, whereas over 65% of CUB-2-coated beads were detached (Figure 4B, open bars). Thus, the bonds formed by CUB-1 were

stronger and more shear resistant than those formed by CUB-2. The data also indicate that the presence of CUB-2 in the CUB-1+2 polypeptide attenuates the interaction with ULVWF.

Because immobilization of either ULVWF or CUB-1 onto a solid surface could artifactually influence their interaction, we also examined the interaction of polypeptides in solution by gel filtration. Based on their vastly different molecular masses, the CUB-1A synthetic peptide and ULVWF should elute in widely separated fractions in a gel filtration column if added individually to the column. However, when mixed and applied to the column, both eluted in the same fractions, indicating that they bind each other (Figure 5). By contrast, when ULVWF was mixed with the scrambled CUB-1A peptide, the peptide eluted alone in the later fractions.

Recombinant CUB-1 polypeptide and synthetic peptides block adhesion of ADAMTS-13 beads to immobilized ULVWF under flow. We next investigated whether the recombinant CUB-1 polypeptide and CUB-1–derived synthetic peptides could interfere with the attachment of ADAMTS-13–coated beads to ULVWF under flow. The beads were incubated with recombinant CUB-1 or any of the synthetic peptides for 3 min before being allowed to settle on the ULVWF surface. After the chamber was perfused with buffer (containing the polypeptide or peptide) for 2 min (shear stress 2.5 dyn/cm²) the adherent beads were counted. In the absence of peptides, 131±18 adherent ADAMTS-13 beads remained per 400X view (Figure 6A). Adhesion was significantly reduced in the presence of either the synthetic CUB-1 peptides (A–E) or the recombinant CUB-1 polypeptide, but not in the presence of the scrambled synthetic peptide.

ULVWF interaction with C-terminal truncated ADAMTS-13. The results presented thus far suggest that CUB-1 binds ULVWF to dock the ADAMTS-13 to the substrate and facilitate its proteolysis in flowing blood. If this were the only mechanism for ADAMTS-13 attachment to ULVWF, one would expect that high concentrations of the polypeptide would be capable of

completely inhibiting both ULVWF proteolysis and ADAMTS-13 bead attachment to ULVWF. In both cases, inhibition appears to reach a plateau. Furthermore, the inhibition data also appear to be in conflict with our earlier observation that a truncated mutant ADAMTS-13 that lacks the sequence C-terminal to the Spacer domain (TSP-2) also cleaves ULVWF strings under flow.³³ One explanation that could resolve these apparent discrepancies is that ADAMTS-13 contains a second binding site for ULVWF that is normally cryptic and can be exposed by truncation of C-terminal sequences. We tested this possibility by perfusing washed platelet suspensions containing either wild-type ADAMTS-13 or TSP-2 mutant over histamine-stimulated endothelial cells and assessing for ULVWF string formation in the presence or absence of recombinant CUB-1 (200 nM). In the absence of CUB-1, both polypeptides completely cleaved ULVWF strings at a shear stress of 2.5 dyn/cm². CUB-1 partially inhibited the cleavage of ULVWF strings by wild-type ADAMTS-13, but had no effect on the activity of the TSP-2 mutant (Figure 6B). Consistent with the functional data, we also detected binding of ULVWF to TSP-2 by ELISA (Figure 6C).

DISCUSSION

We have shown that recombinant CUB-1 polypeptides and synthetic peptides derived from the CUB-1 domain partially inhibited ULVWF cleavage by ADAMTS-13 under flow (Figure 1 and 2). The isolated recombinant CUB-2 domain, in contrast, did not. Neither the recombinant CUB polypeptides nor the synthetic peptides inhibited ULVWF proteolysis under static conditions (Figure 3). We also showed that beads coated with any of the recombinant CUB polypeptides bound directly to ULVWF, but attachment of beads coated with the isolated CUB-2 domain was unable to withstand elevated shear stresses (Figures 4B). Finally, we showed that recombinant CUB-1 inhibits the interaction of ADAMTS-13 with ULVWF to a greater extent under flow than under static conditions (Figure 4). Taken together, these results suggest a critical role for CUB-1 in docking ADAMTS-13 to ULVWF under flow.

In addition to supporting the data generated using recombinant CUB-1, the CUB-1 synthetic peptides also help to define the region of the CUB-1 domain that interacts with ULVWF. Alignment of the CUB1 domain sequence with the sequences of CUB domains of two spermadhesions (Figure 7A) with defined X-ray crystal structures²⁸ shows that the CUB-1 peptides A through D correspond to sequences within the 3rd and 4th β -strands in the known CUB domain structures (Figure 7B). These two β -strands are therefore likely to interact directly with ULVWF. In addition, the peptide CUB-1E, derived from the C-terminus of the CUB-1 domain, also inhibits the binding of ADAMTS-13 to immobilized ULVW to a similar extent as the other four peptides, making it likely that the C-terminal residues are in proximity with the 3rd and 4th β -strands (Figure 7B), and also make up part of the binding interface.

Docking of ADAMTS-13 is necessary to bring the circulating metalloprotease to the ULVWF cleavage site, which is likely to be exposed only transiently when the ULVWF strand

experiences a threshold tensile stress.³⁵ The enzyme would thus be poised to cleave the strand at the appropriate moment when the cleavage site is exposed. This scenario is consistent with our observation that ULVWF strands are most often cleaved near their proximal site of attachment on HUVEC,⁴ the point of maximum tensile stress. Also consistent with this mechanism is the early observation by Tsai and coworkers³⁶ that shear stresses corresponding to those found in human arteries applied to plasma *ex vivo* enhance the proteolysis of VWF. This observation has a pathophysiological correlate: enhanced proteolysis of VWF *in vivo* was shown to produce acquired von Willebrand disease in patients with severe aortic stenosis, a condition in which the blood is continually exposed to elevated shear stress at the site of valve stenosis.³⁷

The conditions in the static assays are very different. In the most commonly used assays, chaotropic agents such as urea are required, presumably to denature the substrate. Under these conditions, one would expect the cleavage site to remain exposed, obviating the need for a docking mechanism. Furthermore, enzyme docking may be less important for cleavage of ULVWF under static conditions because prolonged incubation of VWF and ADAMTS-13 would allow repeated contacts between the substrate and the metalloprotease, the duration of which would be limited only by molecular diffusion and not by fluid flow.

The situation is not that simple, however. Available *in vitro* data are conflicting regarding the role of the CUB domain in the binding of the metalloprotease to VWF. On one hand, CUB domains have been demonstrated to directly bind to VWF³⁸ or modulate the ADAMTS-13-ULVWF interaction.³⁹ Further, Majerus, *et al*³⁹ showed that deletion of the CUB domains reduces ADAMTS-13 binding to VWF three fold. On the other hand, we recently reported that an ADAMTS-13 truncation mutant lacking the sequence C-terminal to the Spacer (without CUB domains) actually cleaved ULVWF more efficiently than wild-type ADAMTS-13 under flow.³³

The same truncation mutant was also found to be active under static conditions.²⁶ What these seemingly contradictory data imply, however, is that ADAMTS-13 contains a second binding site for ULVWF. This presumption is suggested by the data presented in Figure 6.

We therefore propose a working model of ADAMTS-13 activity that takes into account all of the data cited above (Figure 7C). The model posits that ADAMTS-13 in solution is in equilibrium between a closed state in which the second ULVWF-binding domain, and possibly the catalytic site, are cryptic, and an open state in which both the primary and secondary binding sites (and the catalytic site) are available for interacting with the substrate. In this model, the closed conformation is favored when ADAMTS-13 is in solution. However, when ADAMTS-13 binds VWF (through the CUB domains) the equilibrium shifts toward the open conformation (by fluid shear stress for instance), revealing both the second binding site, which reinforces the interaction (note the difference in ULVWF binding between intact ADAMTS-13 and the isolated CUB-1 domain) and the catalytic site. In this way, engagement of the second site is almost always preceded by engagement of the first site, explaining the ability of the isolated CUB-1 domain to inhibit the binding of ADAMTS-13-coated beads to an ULVWF-coated surface. However, ADAMTS-13 may occasionally adopt the open conformation in solution and could therefore bind ULVWF through the usually cryptic second site. This would explain why recombinant CUB-1, even at very high concentrations, cannot completely inhibit ULVWF cleavage (Figures 1 and 2). The second binding site remains to be identified. One likely candidate is the spacer domain, given the findings of Majerus and coworkers³⁹ of a ten-fold drop in the affinity of ADAMTS-13 for ULVWF when the enzyme was truncated before the spacer domain. Other studies have shown that the spacer domain is required for ADAMTS-13 activity under both static and flow conditions.^{26,33}

We wish to emphasize that the proposed interaction between ADAMTS-13 and ULVWF is a working model that was developed based on currently available data, but the existence of open and closed states remain to be further investigated. In addition to this model, other explanations for the data may also be possible. For example, the isolated CUB domains may function distinctively from those in the native enzyme. This is however unlikely for several reasons. First, ULVWF binds similarly to the wild-type ADAMTS-13 and isolated CUB domain polypeptides (Figure 4). Second, tethering and adhesion of ADAMTS-13 coated beads to immobilized ULVWF under flow was blocked by the recombinant CUB-1 polypeptide, as well as the synthetic peptides (figure 2) that are derived from two separate regions of CUB-1. Finally, the inhibitory effect is domain-specific for CUB-1, even though CUB-1 shares a high degree of homology with CUB-2.

In summary, we have demonstrated that the recombinant CUB-1 domain of ADAMTS-13 and peptides derived from it inhibit ULVWF proteolysis under flow conditions, likely by preventing ADAMTS-13 docking to ULVWF. These data highlight an important function for the CUB domains of ADAMTS-13, the only member of the ADAMTS family so far described that contains CUB domains. These results also suggest that ULVWF proteolysis could be impaired by defective docking of ADAMTS-13 to ULVWF under flow (by CUB domain antibodies, for example), a defect that would not be detectable by the conventional static assays currently in use.

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Table 1. Synthetic peptides used in the studies

Name	Sequence	Region
CUB-1A	HLEPTGT	CUB-1 domain
CUB-1As	PELHTTG	Scrambled CUB-1A sequence
CUB-1B	GTIDMRG	CUB-1 domain
CUB-1C	GPGQADCA	CUB-1 domain
CUB-1D	AVAIGRP	CUB-1 domain
CUB-1E	APETFYRE	CUB-1 domain
MET1	GHSFGLEH	Catalytic domain
MET2	GILHLE	Catalytic domain

FIGURES AND LEGENDS

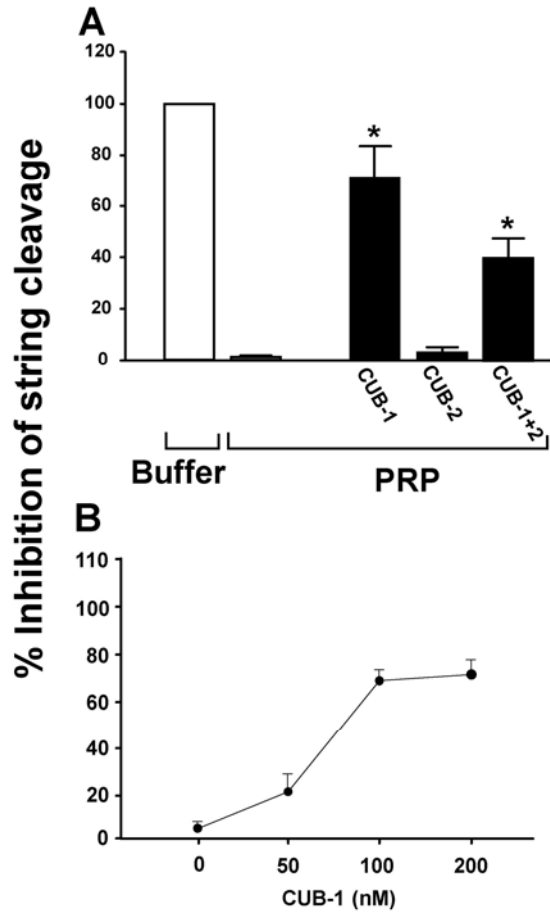


Figure 1. Recombinant CUB-1 polypeptide inhibits UPLWF cleavage under flow conditions.

A. Recombinant CUB polypeptides (100 nM) were incubated with PRP for 10 min and then perfused over histamine-stimulated HUVECs at 2.5 dyn/cm² of shear stress. UPLWF strings formed when buffer was perfused (0% activity), but were cleaved when PRP was perfused (100% activity). The UPLWF strings were detected in PRP that was pretreated with CUB-1 or CUB-1+2, but not when pretreated with CUB-2. **B.** The inhibition of UPLWF cleavage by CUB-1 polypeptide was dose-dependent with maximal inhibition at 100 nM. The data are mean \pm SEM (Student's *t* test, * < 0.01 compared to untreated samples).

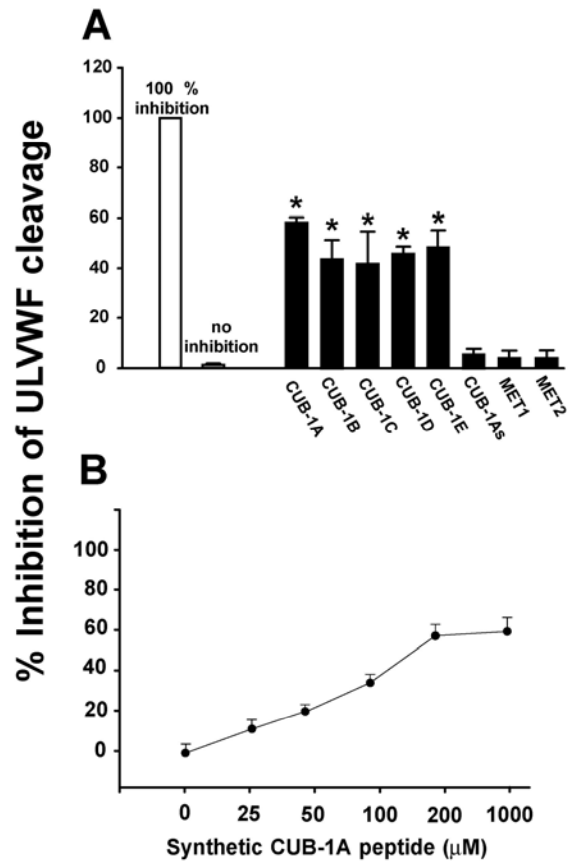


Figure 2. Synthetic CUB-1 peptides block the cleavage of ULVWF under flow. **A.** Each of the synthetic peptides (final concentration 200 μM) was incubated with PRP for 10 min at room temperature and the mixture was then perfused over the histamine-stimulated endothelial cells. All of the peptides corresponding to CUB sequences (CUB-1A to E) partially inhibited the cleavage of ULVWF strings, but the scrambled peptide CUB-1As did not. Two peptides corresponding to sequences in the catalytic domain (Met1 and Met2) also did not inhibit cleavage. **B.** Inhibition of proteolysis by recombinant CUB-1 was dose-dependent with a maximal effect at 200 μM . The data are presented as mean \pm SEM (Student's *t* test, * < 0.01 compared to untreated samples).

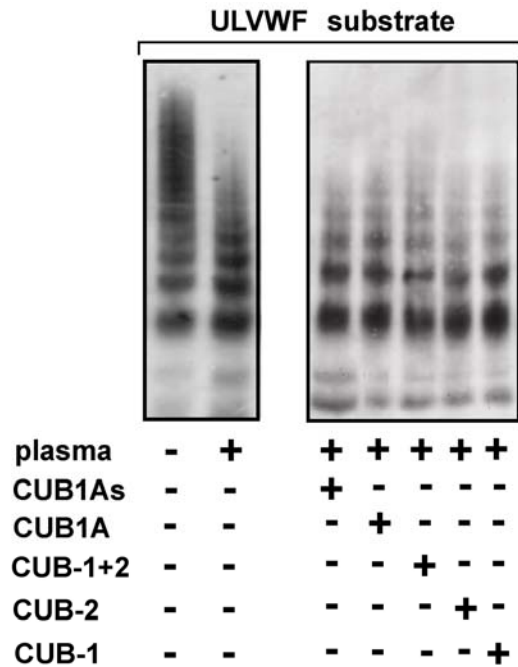


Figure 3. Neither recombinant CUB domains nor a CUB-1 synthetic peptide inhibit the cleavage of ULVWF under static conditions. ULVWF was incubated with barium-treated plasma in the presence of recombinant polypeptides (CUB-1, CUB-2, and CUB-1+2) or synthetic peptide (CUB1A) or its scrambled form (CUB1As) for 24 hr in the presence of 1.5 M urea. The cleavage of ULVWF was then assessed by 1% agarose gel electrophoresis and immunoblotting. Plasma ADAMTS-13 cleaved the ULVWF multimers and the cleavage was not affected by pretreatment of plasma with any of the CUB polypeptides or synthetic peptides. The figure is a representative of three separate experiments.

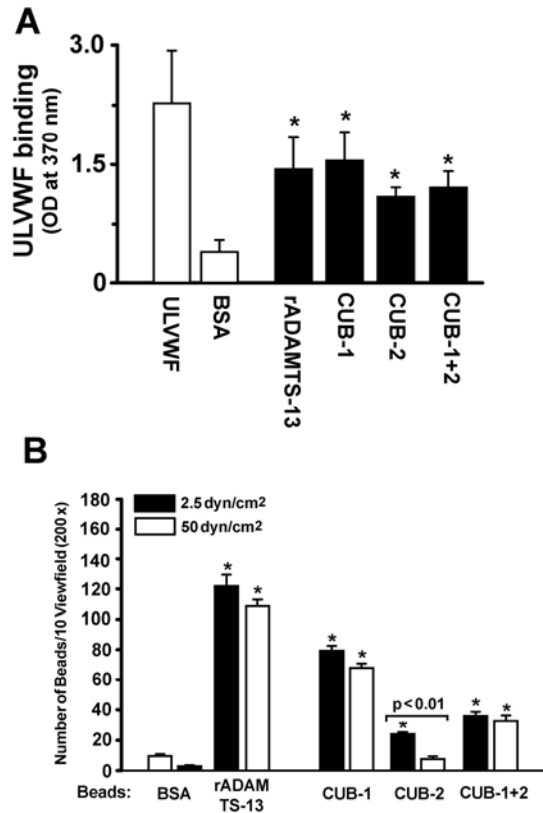


Figure 4. ULVWF binds recombinant CUB polypeptides under static and flow conditions:

A. Static assay: ULVWF was incubated with immobilized wild-type ADAMTS-13 or CUB domain polypeptides for 30 min and bound ULVWF was detected using a polyclonal VWF antibody. The data are expressed as mean \pm SEM (Student's *t* test, *n* = 5, * *p* < 0.001 for each peptide compared to BSA). **B. Flow assay:** Polystyrene beads coated with ADAMTS-13 or CUB polypeptides were allowed to settle on immobilized ULVWF and then perfused with buffer for 2 min. The beads remaining adherent were counted (back bar). The wall shear stress was then increased to 50 dyn/cm² for 2 min perfusion and beads remained were again counted (open bar). The data are mean \pm SEM of beads bound in 10 random 400X review fields (Student's *t* test, *n* = 4, * *p* < 0.001 compared to BSA beads).

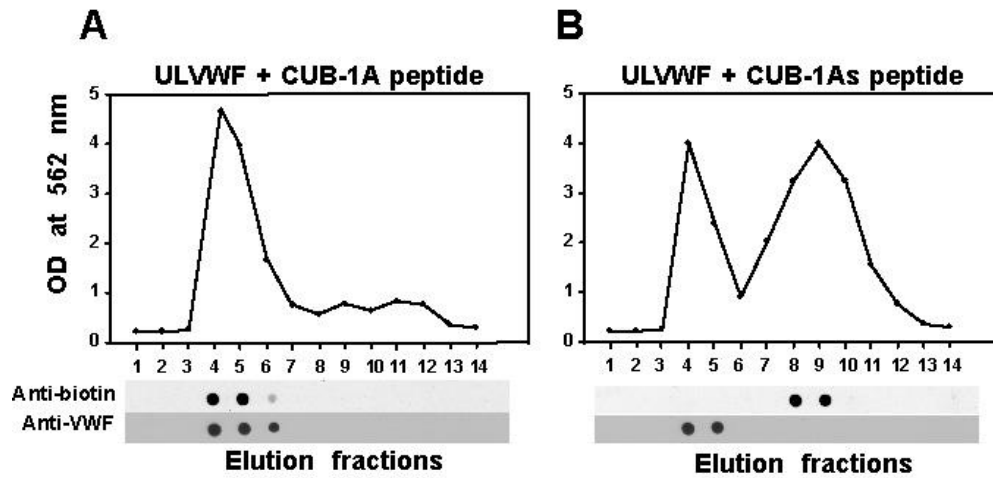


Figure 5. *The CUB-1A peptide co-elutes with ULVWF from a gel filtration column.*

Biotinylated CUB-1A peptide and CUB-1As were incubated with ULVWF for 15 min and then allowed to pass through a gel filtration column at a flow rate of 1 ml/min. The eluate was collected in 14 fractions of 1 ml each and probed for the peptide with a polyclonal anti-biotin antibody and for ULVWF with a monoclonal VWF antibody. The CUB-1A peptide co-eluted with ULVWF (**A**), whereas the CUB-1As peptide did not (**B**). The figure is a representative of 3 separate experiments.

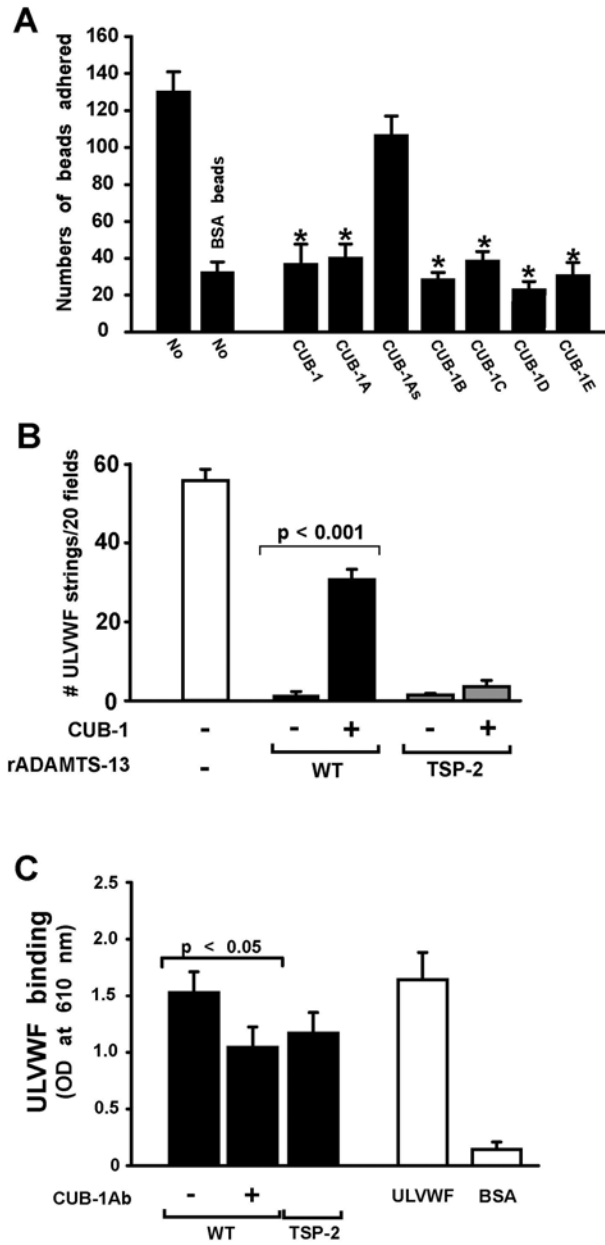


Figure 6. CUB-1 peptides blocked ADAMTS-13 binding to immobilized ULVW. **A.** Beads coated with ADAMTS-13 were perfused over immobilized ULVWF in the presence or absence of recombinant CUB-1 polypeptide (CUB-1, 100 nM) or synthetic peptides (CUB-1A-E, 200 μ M). ADAMTS-13 beads adhered to immobilized ULVWF and the adhesion was blocked by the recombinant CUB-1 polypeptide and CUB-1A-E peptides, but not by the CUB-1As polypeptide (Student's *t* test, $n = 4$, $* < 0.01$ compared to untreated ADAMTS-13- beads). **B.**

Washed platelets were perfused over histamine-activated HUVEC in the presence of either WT ADAMTS-13 or the truncation mutant TSP-2. The numbers of strings detected after 2 min perfusion was counted. CUB-1 polypeptide partially blocked the cleavage of ULVWF by WT ADAMTS-13 as demonstrated by the increased numbers of ULVWF strings, but not by the TSP-2 truncation mutant (Student's *t* test, $n = 4$). **C.** Binding of ULVWF to wild-type ADAMTS-13 or TSP-2 mutant was measured by ELISA. ULVWF bound WT ADAMTS-13 and TSP-2 mutant. The binding to WT ADAMTS-13 can be partially blocked by an anti-ADAMTS-13 antibody (10 $\mu\text{g/ml}$) generated using as an immunogen a synthetic peptide from the CUB-1 domain (Student's *t* test, $n = 7$). All data are expressed as mean \pm SEM.

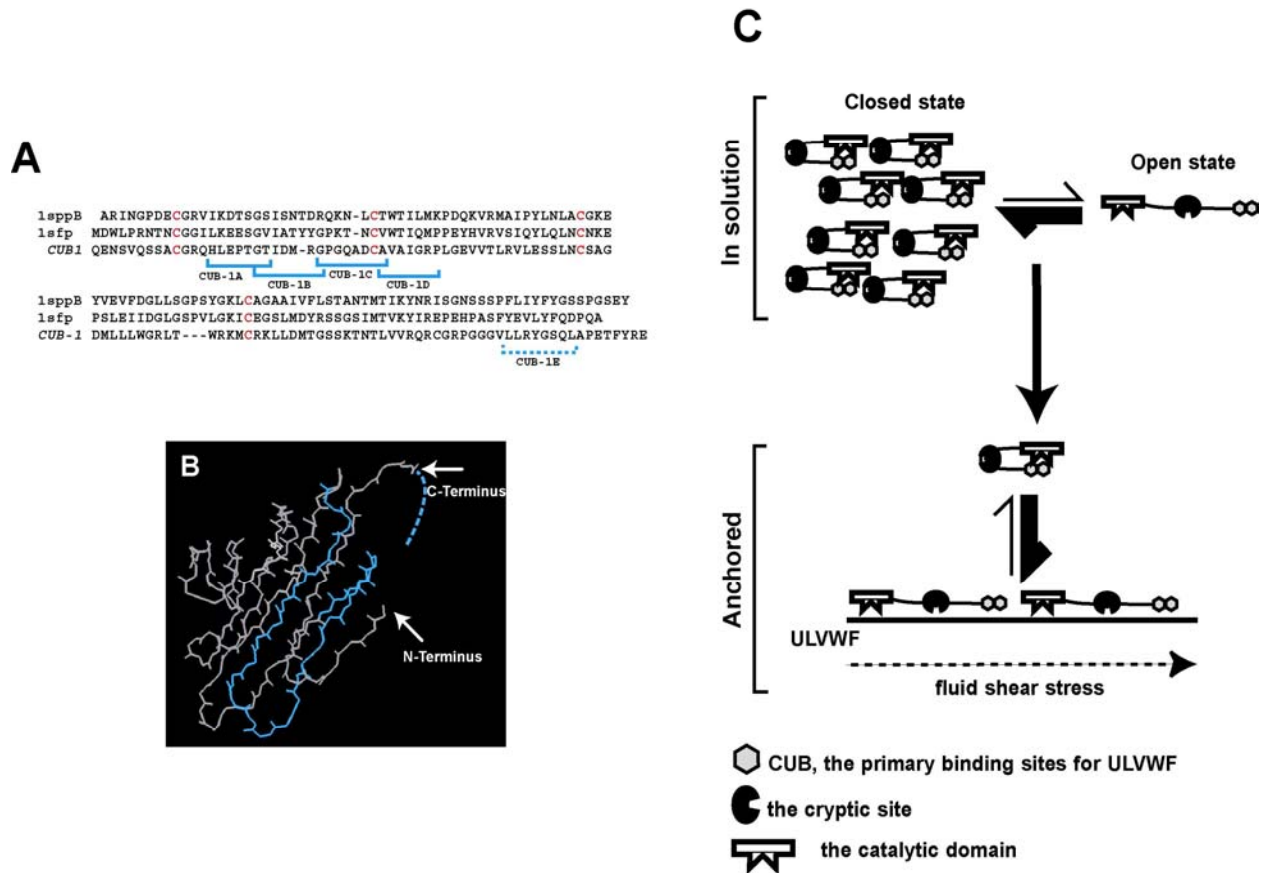


Figure 7. A model of two-site binding of ADAMTS-13 to ULVWF. **A.** Alignments of the sequences of the CUB-1 domain of ADAMTS-13 and the CUB domains of two spermadhesins. **B.** Four of the five peptides were located in the 3rd and 4th β -strands of the CUB fold, indicating that this region is involved in binding ULVWF. One peptide was derived from the C-terminus that potentially interacts with the 3rd and 4th β -strands. **C.** A two-site model of ADAMTS-13 binding to ULVWF. ADAMTS-13 exists in plasma in open and closed states that are in equilibrium with the closed state being the dominant form. The binding of ADAMTS-13 through its CUB-1 domain to ULVWF anchored to the surface of endothelial cells is facilitated by fluid shear stress. Once bound, ADAMTS-13 assumes the open conformation that exposes the second cryptic binding site to stabilize the interaction with ULVWF.