

Enzymatically Active ADAMTS13 Variants Are Not Inhibited by Anti-ADAMTS13 Autoantibodies

A NOVEL THERAPEUTIC STRATEGY?*

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ADAMTS13 (a disintegrin and metalloprotease with thrombospondin motifs), a circulating multidomain zinc metalloprotease of the reprotolysin subfamily, is critical for preventing von Willebrand factor-platelet interaction under high shear stress conditions. A deficiency of the protease, due to mutations in the *ADAMTS13* gene or the presence of antibodies that inhibit the activity of the protease, causes thrombotic thrombocytopenic purpura (TTP). Plasma therapy, the conventional therapy for TTP, may cause serious adverse reactions and is ineffective in some patients. In order to develop new strategies for improving the diagnosis and treatment of TTP, we produced a series of truncated ADAMTS13 proteins in mammalian cells and analyzed their binding with and suppression by the IgG derived from the TTP patients. The results revealed that truncation of the ADAMTS13 protein at its cysteine-rich region eliminated its recognition by the antibodies without abolishing its von Willebrand factor-cleaving activity. This raises the possibility that resistant ADAMTS13 variants may be exploited to circumvent inhibitory antibodies that cause TTP.

von Willebrand factor (VWF),² a multimeric hemostatic glycoprotein, is secreted from vascular endothelial cells as an ultralarge, disulfide-bonded polymer of 2050 amino acid residues (1). In the circulation, this large polymer undergoes shear-dependent cleavage at the Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ bond in its A2 domain by a plasma zinc metalloprotease, ADAMTS13 (a disintegrin and metalloprotease with thrombospondin motifs), to become a series of multimers (2). This cleavage of VWF is critical for preventing unwanted intravascular VWF-platelet binding, and a deficiency of ADAMTS13 causes microvascular platelet thrombosis that is characteristic of thrombotic thrombocytopenic purpura (TTP) (3).

TTP is a relatively uncommon but serious disease that, if untreated, causes death in greater than 90% of the affected cases (4). In the majority of patients, neutralizing autoantibodies against the protease cause its deficiency (5–9). In a small subset of patients, ADAMTS13 deficiency is associated with mutations of the *ADAMTS13* gene (Upshaw-Schulman

syndrome) (10–19). The molecular mechanism of ADAMTS13 deficiency is a critical determinant of a patient's response to plasma therapy. Patients with mutational deficiency of ADAMTS13 typically achieve remission with 10–15 ml of fresh frozen plasma per kg of body weight administered every 2–3 weeks. In contrast, patients with inhibitory autoantibodies of the protease require plasma exchange for treatment. This therapy uses an apheresis machine to replace the entire volume of the body's plasma with normal human plasma (20). In order to maintain adequate protease levels, the procedure is commonly repeated daily for days to weeks. Plasma exchange therapy is expensive, technically demanding, and ineffective for patients with high or persistent inhibitory autoantibodies.

ADAMTS13 is a multidomain zinc metalloproteinase that belongs to the reprotolysin subfamily of the metalloproteinase M12 family (21). In order to develop new strategies for improving the diagnosis and treatment of TTP, this study systemically analyzed a series of ADAMTS13 mutant proteins to identify variant forms that are proteolytically active and yet resistant to suppression by inhibitory antibodies.

MATERIALS AND METHODS

Plasmid Constructs—The DNA sequences for the various recombinant ADAMTS13 variants were generated by PCR using a plasmid construct (pCDNA3.1-ADAMTS13Full2-2) as the template. This construct contained the entire coding sequence of the human *ADAMTS13* gene (GenBankTM accession number AF414401) (10) but with the 5'-untranslated sequence deleted and replaced with an optimized Kozak consensus sequence (uppercase), 5'-tcgatcctc-gagtctagaGCCGCCACCATG, with the underlined ATG serving as the translation initiation codon. For the AD1–AD7 variants (Fig. 1), the relevant regions of the ADAMTS13 sequence were amplified and inserted into a mammalian expression vector, pCDNA3.1/V5-His-TOPO (Invitrogen). For the AD8–AD13 variants, the relevant regions were amplified and inserted into the vector pSecTag/FRT/V5-His-TOPO (Invitrogen). The primer pairs used for amplification of the ADAMTS13 sequences are listed in TABLE ONE. All PCRs used PfuUltraTM Hotstart DNA Polymerase (Stratagene, La Jolla, CA), with thermocycling at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1–4 min, and ending with 72 °C for 10 min. Then a single deoxyadenosine (A) was added to the 3'-ends of PCR products by using TaqDNA polymerase (Invitrogen). All constructs were confirmed for accuracy by DNA sequencing. For the AD1–AD7 variants, this cloning strategy added a vector-encoded amino acid sequence to the carboxyl terminus of the ADAMTS13 sequences, consisting of a linker (KGN-SADIQHSGGRSSLEGPRFE), the V5 sequence (GKPIPNPLLGLDST), a tripeptide (RTG), and a His₆ tag. For the AD8–AD13

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² The abbreviations used are: VWF, von Willebrand factor; GST, glutathione S-transferase; NHP, normal human plasma; TSP1, thrombospondin-1 motif 1; TSP2 to -8, thrombospondin-1 motifs 2–8; TTP, thrombotic thrombocytopenic purpura; ELISA, enzyme-linked immunosorbent assay.

TABLE ONE

Oligonucleotide primers used for amplification of the ADAMTS13 cDNA

Variant	Forward primer sequence	Reverse primer sequence
AD1	gctcggatccactagtccag	ctgctcgttggcgtgta
AD2	gctcggatccactagtccag	ttcgacatgaactccagc
AD3	gctcggatccactagtccag	agcaccctcagtgtagaagg
AD4	gctcggatccactagtccag	gcactgctgttgtcccccac
AD5	gctcggatccactagtccag	gtagggagggcagggttc
AD6	gctcggatccactagtccag	cgactgacttgagagaac
AD7	gctcggatccactagtccag	ggttccttcttcccttc
AD8	agccacggaagggtct	gtagggagggcagggttc
AD9	cagtgcgccaggaccgacgg	gtagggagggcagggttc
AD10	atcaaccctgaggacgacac	gtagggagggcagggttc
AD11	atcaaccctgaggacgacac	gcactgctgttgtcccccac
AD12	gaggagtatggcaacctacc	cgactgacttgagagaac
AD13	tggaaagtcatgtccttgg	ggttccttcttcccttc

variants, the strategy introduced an Ig κ -chain secretion signal peptide (METDTLLLVLLLWVPGSTGD) and a linkage peptide (AAQPARRARRTKLAL) at the amino terminus, and a vector amino acid sequence consisting of a linker (KGELGTELGSE) and the V5 and His₆ tags at the carboxyl terminus.

Recombinant Protein Expression—COS-7 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin, and streptomycin. One day after subculture, the cells at 90% confluence were transfected with plasmid DNA using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The culture media were changed to serum-free media the next day and collected after 72 h. After the cell debris was removed by centrifugation, the samples were concentrated 15-fold using a Centricon YM-10 concentrator (Millipore, Billerica, MA) and stored at -70°C . The adherent cells and matrix were detached from culture dishes and lysed with SDS-PAGE sample buffer.

Recombinant proteins were separated by SDS-PAGE and visualized by immunoblotting using a monoclonal anti-V5 antibody (Invitrogen), a horseradish peroxidase-conjugated anti-mouse IgG, and the SuperSignal chemiluminescent substrate (Pierce). The luminograms were scanned, and the relative amounts of proteins were estimated by densitometry using ImageQuant (Amersham Biosciences).

Analysis of ADAMTS13 Activity—The ADAMTS13 activity levels in culture media or plasma samples were analyzed by using a previously developed assay (22). In the assay, VWF multimers isolated from normal fresh frozen human plasma and treated with 1.5 mol/liter guanidine HCl were used as the substrate. Measurement of ADAMTS13 activity was based on the levels of VWF proteolytic fragments that were generated, as determined densitometrically on immunoblots using the ImageQuant software (22). We also used a recombinant human VWF fragment (GST-VWF73-His), which contained 73 amino acids (Asp¹⁵⁹⁶–Arg¹⁶⁶⁸) of VWF and was flanked at the amino and carboxyl termini, respectively, by glutathione *S*-transferase (GST) and hexahistidine sequences as a substrate. Cleavage of substrates by the recombinant ADAMTS13 proteins in the cell culture-conditioned media was performed in solution, with products of the reaction separated by SDS-PAGE and identified by immunoblotting using a monoclonal anti-GST antibody or by an enzyme-linked immunosorbent assay (ELISA) as described previously (23).

For the ADAMTS13 inhibition assay, the cell culture-conditioned media were incubated at room temperature for 30 min with three vol-

umes of heat-inactivated human plasma samples diluted to different degrees before the ADAMTS13 activity level was measured (24).

Patient Plasma Samples—Sodium citrate anticoagulated blood samples were obtained by venipuncture from patients with a diagnosis of TTP at the time of initial presentation or before each session of plasma exchange. After centrifugation to remove blood cells, the plasma samples were collected and stored at -70°C . For mixing studies, aliquots of the plasma samples were incubated at 56°C for 60 min. After centrifugation to remove the insoluble components, the heat-treated plasma samples were saved at -70°C . All patients were confirmed to have less than 0.1 unit/ml ADAMTS13 protease activity levels at the time of presentation and had inhibitors of the protease. An institutional review committee approved the study protocol.

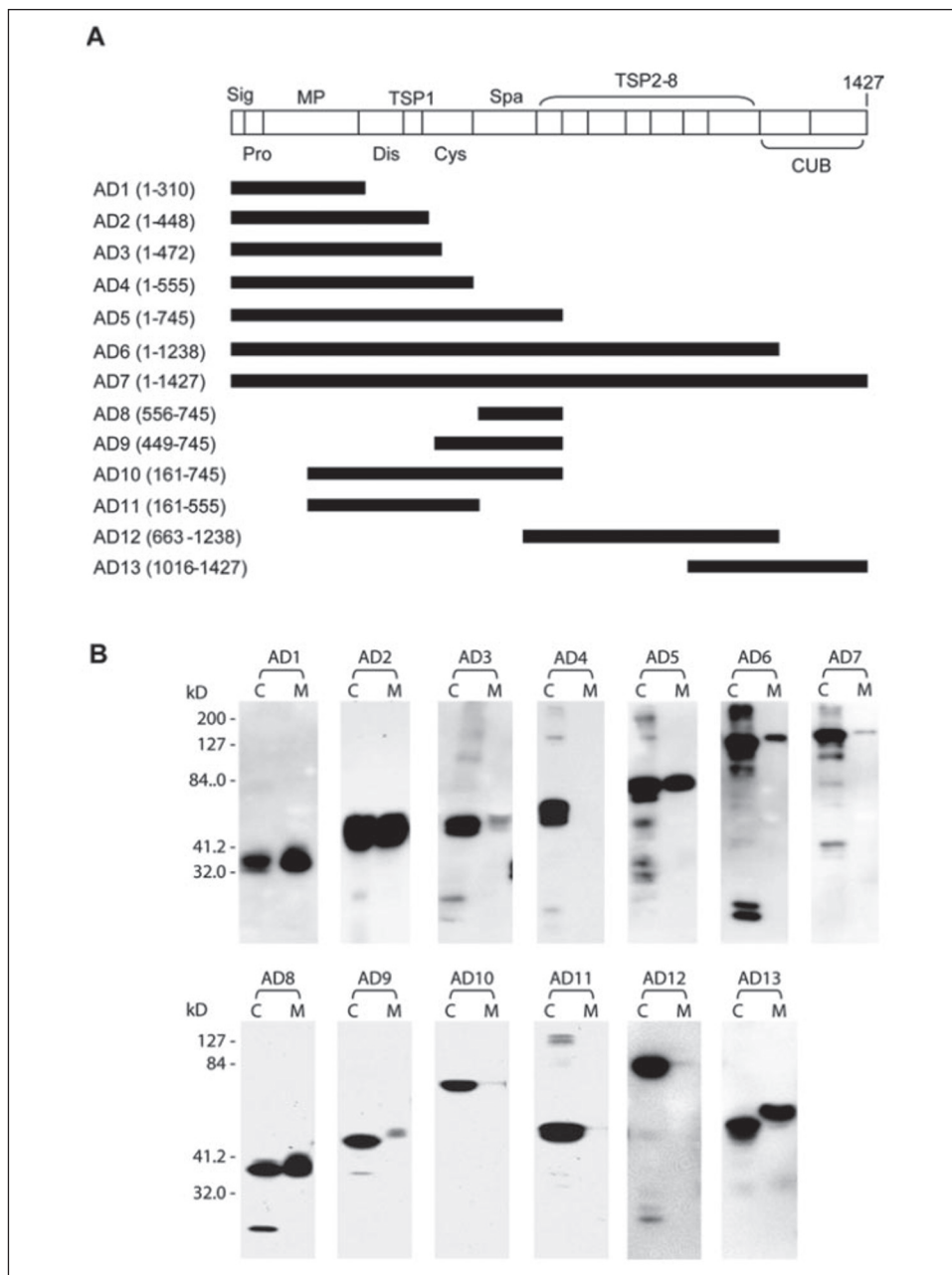
Preparation of ADAMTS13 Affinity Column—ADAMTS13 protein was isolated from fresh frozen human plasma as previously described (25). The protein, containing 1.5 units of protease activities/mg of protein, was immobilized with cyanogen bromide-activated Sepharose 4B-agarose beads (Sigma). The beads were poured into a column and stored at 4°C in Tris-saline (50 mmol/liter Tris, 50 mmol/liter NaCl, pH 8.0, and 0.02% sodium azide).

Isolation of ADAMTS13 Antibodies—IgG molecules isolated from heat-inactivated human plasma samples using staphylococcal protein A-agarose beads (Pierce) were applied to an ADAMTS13 affinity column and then eluted with 0.1 mol/liter glycine, 0.5 mol/liter NaCl, pH 2.8. The glycine buffer eluates were immediately neutralized with one-tenth volume of 1 mol/liter Tris/HCl, pH 8.8, and one-tenth volume of 2 mg/ml bovine serum albumin. After dialysis against Tris-saline, the IgG proteins were concentrated to the original plasma volume. The protein solution used in this study contained 1.2 $\mu\text{g/ml}$ IgG, as determined by an IgG assay kit (Pierce), and 213 units of protease-inhibiting activity/mg of IgG protein.

Immunoprecipitation of ADAMTS13 Proteins—To determine whether an ADAMTS13 protein binds to the IgG molecules of patients with TTP, 200 μl of IgG purified using an ADAMTS13 affinity column or protein A-agarose beads was incubated with 20 μl of protein A-coated agarose beads at room temperature for 1 h. After washing with Tris-saline buffer (50 mM Tris and 50 mM NaCl, pH 8.0), the immunobeads were incubated with 20 μl of a concentrated cell culture-conditioned medium at room temperature for 1 h. The supernatant was collected by centrifugation and concentrated to 10 μl on Microcon concentrators. After the agarose beads were washed with Tris saline

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FIGURE 1. Design and production of recombinant ADAMTS13 proteins. A, schematic depiction of the domain structure of ADAMTS13 and the forms of variant ADAMTS13 proteins used in this study. For each construct, the numbers in the parenthesis indicate the segment of amino acid residues of ADAMTS13. *Sig*, signal peptide; *Pro*, propeptide; *MP*, metalloprotease; *Dis*, disintegrin; *TSP1*, thrombospondin-1 motif 1; *Cys*, cysteine-rich region; *Spa*, spacer; *TSP2-8*, thrombospondin-1 motifs 2–8; *CUB*, cub domains. B, production of proteins in COS-7 cells. Cells were transiently transfected with plasmid constructs for various ADAMTS13 variants. Conditioned medium was concentrated 15-fold, whereas the cells were lysed with SDS-PAGE sample buffer. A monoclonal anti-V5 antibody was used to detect levels of the ADAMTS13 proteins in cell lysates (C) and in the culture media (M) by immunoblotting.



buffer, the bound proteins were eluted with 20 μ l of SDS sample buffer in the presence of 5% 2-mercaptoethanol.

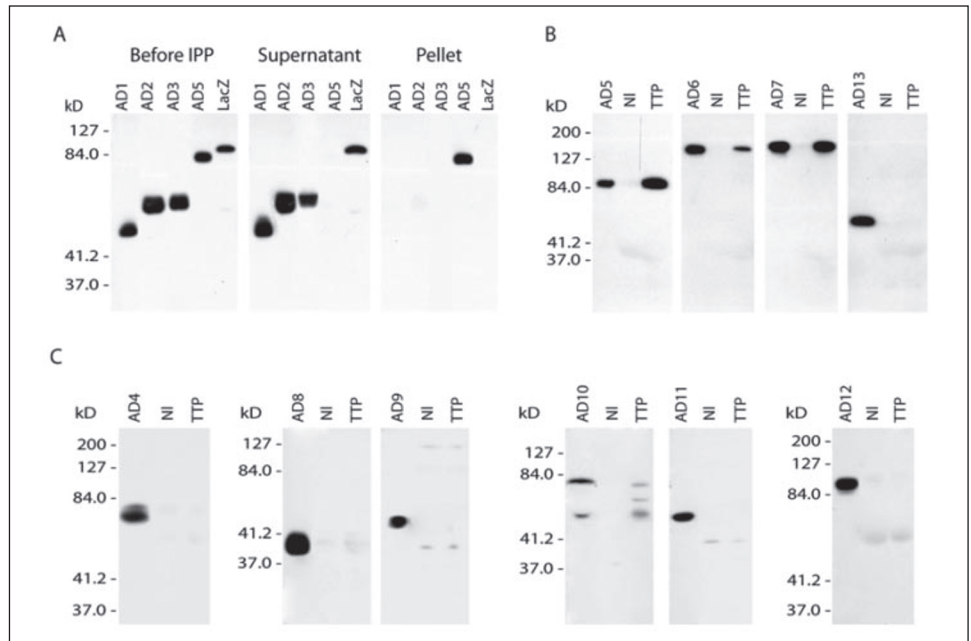
Detection of Binding between ADAMTS13 Proteins and IgG by ELISA—Polyclonal species-specific goat anti-mouse IgG (40 μ g/ml) was immobilized onto microtiter plate wells in the presence of sodium bicarbonate coating buffer (pH 9.6). After blocking with 1% casein buffer, pH 8.0 (Bio-Rad), the plates were incubated successively with a monoclonal anti-V5 antibody at a 1:1000 dilution for 1 h, cell culture-conditioned media containing ADAMTS13 proteins for 1 h, plasma samples at various dilutions for 1 h, biotinylated, polyclonal species-specific goat anti-human IgG for 1 h, and avidin-conjugated horseradish peroxidase for 1 h. Between incubation steps, the plates were washed three times with 25 mmol/liter Tris/HCl, pH 7.5, 150 mmol/liter NaCl, and 0.01% Tween 20. Bound IgG was detected with the chromogenic substrate 5'-aminosalicylic acid. The OD values were read at 450 nm. For blocking experiments,

the plasma samples from TTP patients were incubated with cell culture-conditioned media containing untagged full-length ADAMTS13 at 10 units/ml before use in ELISA.

RESULTS

Production of Full-length and Truncated Recombinant ADAMTS13—Fig. 1A depicts the domain structure of the full-length ADAMTS13 protein and the forms of the several truncated variants of the protein that we generated for this study. The AD7 form represents the full-length human ADAMTS13 with the published complete coding sequence (GenBankTM accession number AF414401). Variants AD1–AD6 were each truncated at a site upstream of the carboxyl terminus, whereas AD8–AD13 each contained a segment of the ADAMTS13 protein downstream of the amino terminus. All recombinant proteins were produced in COS-7 cells. To facilitate the study, a V5 epitope tag was added to the carboxyl terminus of the

FIGURE 2. Immunoprecipitation of ADAMTS13 proteins with TTP IgG. *A*, immunoprecipitation with ADAMTS13 affinity-purified TTP IgG. *Left panel*, four ADAMTS13 variant proteins (AD1, AD2, AD3, and AD5) and an irrelevant protein, β -galactosidase (LacZ), as detected with anti-V5 antibody before immunoprecipitation (IPP). *Middle panel*, AD5 was depleted from the supernatant after the immunoprecipitation. *Right panel*, only AD5 was present in the pellets of immunoprecipitation. *B* and *C*, immunoprecipitation of ADAMTS13 variant proteins AD4–AD13 with TTP IgG. For each protein, the *panel* includes *three lanes*: the protein before immunoprecipitation, the protein in the pellets of normal IgG/protein A beads (NI), and the protein in the pellets of TTP IgG/protein A beads (TTP). AD5, AD6, AD7, and AD10 were detected in the pellets of TTP IgG. None of the proteins were in the pellets of normal IgG.



recombinant proteins. A signal peptide was also added to allow secretion of the amino terminus truncated mutants. Immunoblotting with a monoclonal anti-V5 antibody revealed that the truncated versions of ADAMTS13 were synthesized and secreted in the culture media at variable levels (Fig. 1*B*). Variants AD4, AD10, AD11, and AD12 were barely detectable in the culture media, as shown, but their presence became clear after prolonged exposure (not shown).

Analysis of Binding between IgG from TTP Patients and Recombinant ADAMTS13 Proteins by Immunoprecipitation—Since preliminary experiments showed that immunoglobulins from TTP patients (TTP IgG) reacted poorly with the ADAMTS13 protein on Western blots, we used an immunoprecipitation method to investigate the binding sites of TTP IgG on ADAMTS13. Truncated ADAMTS13 variants AD1, AD2, AD3, and AD5 were incubated with the affinity-purified IgG fraction of a TTP patient's plasma and precipitated with protein A-coated agarose beads. The presence of a recombinant protein in the samples was detected by Western blotting using a monoclonal anti-V5 antibody. V5-tagged recombinant β -galactosidase protein was also included as a control. The results shown in Fig. 2*A* demonstrate that among the five recombinant proteins used in the immunoprecipitation analysis, variant AD5 was depleted from the supernatant and enriched in the pellet, whereas the other variants were unaffected. We performed immunoprecipitation analysis of variants AD4–AD13 with TTP IgG in similar but separate experiments using normal IgG as a negative control (Fig. 2, *B* and *C*). The analysis revealed that variants AD5, AD6, AD7, and AD10, but not AD4, AD8, AD9, AD11, AD12, or AD13, were present in the TTP IgG pellets. None of the proteins were detected in the pellets with normal IgG, indicating that the precipitation caused by TTP IgG was not due to nonspecific adsorption. These results suggest that the region of ADAMTS13 protein between residues 161 and 745 contains all of the epitopes necessary for the binding of the inhibitory antibodies in the plasma of TTP patients.

Detection of Binding between TTP IgG and ADAMTS13 Proteins by ELISA—To extend this study to a larger number of IgG fractions from TTP patients, we designed an ELISA-based binding assay. Microtiter plate wells were coated with goat anti-mouse IgG and then with a monoclonal anti-V5 antibody. The plates coated with monoclonal anti-V5 were used to capture each of the recombinant ADAMTS13 variant proteins in the cell culture-

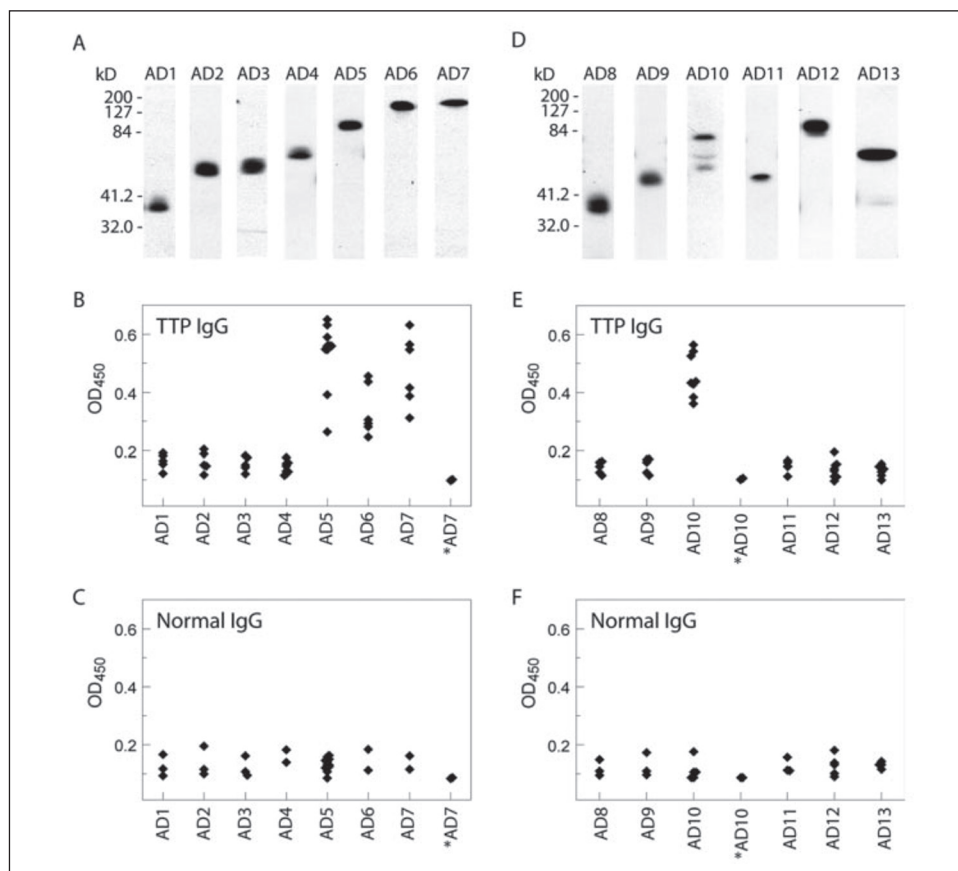
conditioned media, adjusted to similar concentrations as evidenced by Western blotting (Fig. 3, *A* and *D*). The plates coated with the ADAMTS13 variant proteins were then incubated with heat-treated plasma samples from TTP patients (Fig. 3, *B* and *E*) or normal humans (Fig. 3, *C* and *F*). After washing, the presence of human IgG on the plate was detected with an anti-human IgG antibody as described under "Materials and Methods." In preliminary experiments using 13 normal plasma samples, the mean OD value \pm S.D. was 0.132 ± 0.028 against AD5, which was not different from the mean OD value of 0.137 ± 0.002 against the control casein protein alone. Based on these results, we set the threshold OD level for a positive reaction at 0.220. Using this criterion, each of the TTP IgGs tested exhibited positive binding with variant AD5, AD6, AD7, or AD10, but not with AD1, AD2, AD3, AD4, AD8, AD9, AD11, AD12, or AD13 (Fig. 3, *B* and *E*). None of these variant proteins capture normal IgG above the background values (Fig. 3, *C* and *F*). Overall, the binding data detected with ELISA was in accordance with the results of immunoprecipitation analysis described in Fig. 2.

To exclude the possibility that the observed binding was directed against an exogenous epitope of the tagged ADAMTS13 protein that is absent in native ADAMTS13, we incubated two randomly selected TTP plasma samples with recombinant, untagged, full-length ADAMTS13 protein before applying them to the microtiter plate wells containing immobilized AD7 or AD10 variants. The results showed that incubation with untagged ADAMTS13 completely blocked the binding of TTP IgG to either AD7 or AD10 (Fig. 3, *B* (*AD7) and *E* (*AD10)), confirming that the observed binding was specific for ADAMTS13 protein.

Analysis of VWF-cleaving Activity and Its Suppression by TTP IgG—We then explored whether the truncated versions of ADAMTS13 that were not recognized by the TTP IgG retained proteolytic activity. The ADAMTS13 proteins were incubated with heat-treated TTP plasma or control normal human plasma before their proteolytic activity in cleaving VWF was measured. Each ADAMTS13 variant was incubated with the VWF substrate in the absence or presence of EDTA. In the absence of EDTA, ADAMTS13 cleaved VWF at the Y1605-M1606 bond, generating homodimers of 176- and 140-kDa fragments (labeled (176kD)₂ and (140kD)₂), which were detected by immunoblotting using a polyclonal anti-VWF antibody after nonreducing SDS-PAGE. EDTA

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FIGURE 3. Binding between the ADAMTS13 variants and TTP IgG. *A* and *D*, immunoblots of the recombinant proteins used in the binding experiments. The recombinant proteins were detected by using monoclonal anti-V5. *B*, *C*, *E*, and *F*, recombinant ADAMTS13 proteins immobilized onto monoclonal anti-V5 coated microtiter plate wells were incubated with either TTP or normal human plasma. The captured human IgG were detected chromogenically as described under "Materials and Methods." Each *symbol* represents the result with one TTP or normal sample. Each protein was tested against 5–9 different TTP and 2–7 normal plasma samples. Compared with normal plasma, TTP plasma contained IgG molecules with positive binding to AD5, AD6, AD7, and AD10 but not to AD1–AD4, AD8, AD9, or AD11–AD13. To confirm the specificity of the observed binding, the TTP samples were also tested against AD7 or AD10 after incubation with untagged recombinant full-length ADAMTS13, which blocked the binding of TTP IgG to AD7 or AD10 (marked as *AD7 and *AD10).



suppressed the protease activity responsible for generating these species. Visual analysis of the blots showed that variants AD2, AD3, AD5, AD6, and AD7, but not AD1, were enzymatically active, generating the expected VWF fragments in the absence of EDTA (Fig. 4A). Intriguingly, incubation of the proteases with heat-treated TTP plasma suppressed the proteolytic activity of variant AD5, AD6, or AD7 but not AD2 or AD3 (Fig. 4B).

To determine whether other TTP plasma samples also suppressed the VWF-cleaving activity of the ADAMTS13 proteins, we incubated variants AD2 or AD7 (full-length ADAMTS13) with plasma samples isolated from nine additional TTP patients and found that each of the TTP samples suppressed the VWF-cleaving activity of AD7 but not of AD2 (Fig. 4, C and D). In separate experiments, we detected 0.15–0.2 units/ml VWF-cleaving activity in AD4 but no activity in variants AD8–AD13 or in an irrelevant protein (β -galactosidase) and found that TTP plasma also did not suppress the activity of AD4 (data not shown).

We then determined whether the truncated ADAMTS13 variants cleaved a VWF fragment (GST-VWF73-His) that consisted of 73 amino acids spanning the Tyr¹⁶⁰⁵–Met¹⁶⁰⁶ cleavage site of VWF and has been used as a substrate in diagnostic assays (23). Cleavage of the substrate by ADAMTS13 decreased its size from 38.1 to 30.4 kDa, which was detected using a monoclonal anti-GST antibody in immunoblots (Fig. 5). The results showed that normal human plasma (NHP) and variants AD2, AD3, AD5, AD6, or AD7, but not AD1, cleaved GST-VWF73-His (Fig. 5A). After incubation with a TTP plasma, similar cleavage of the substrate was detected with AD2 or AD3, but not with AD5, AD6, or AD7, indicating that the TTP plasma suppressed the activity of variants AD5–AD7 but not of AD2 or AD3 (Fig. 5B). Similar results were obtained when the assay of GST-VWF73-His cleavage was conducted in

an ELISA format; TTP plasma suppressed the activity of variants AD2 or AD3, but not of AD5, AD6, or AD7 (Fig. 5C).

TABLE TWO summarizes the VWF-cleaving activities, their suppression by TTP plasma, and the binding properties, as detected by immunoprecipitation methods and ELISA, of the variants.

Comparison of Relative Protease Activity—To compare the relative catalytic activities of the ADAMTS13 variants, we calculated the protease/concentration ratio of each ADAMTS13 variant. The protein concentrations were measured by extrapolating the OD values of the signals from anti-V5 immunoblots against a reference curve generated using a serially diluted AD5 sample. Each of the measured values was then expressed as a fraction or multiple of the concentration of AD7 (TABLE THREE). The relative catalytic activities of variants AD5 and AD6, after adjustment for the respective protein concentrations, were ~40–75% of AD7. In contrast, the relative catalytic activity of AD2 or AD3 was less than 1–2% of AD7. The levels of AD4 were too low for this analysis.

TTP Plasma Did Not Suppress the Protease Activity of AD2—To determine whether AD2 was consistently resistant to suppression throughout the course of an acute TTP episode, we added variant AD2, or AD7 as a control, to serial plasma samples obtained from a patient with varying inhibitory autoantibody levels (Fig. 6). This patient presented with typical features of TTP and had an inhibitor titer of 5 units/ml. She had a transient improvement in response to plasma exchange, with the platelet count reaching 160×10^9 /liters on days 6 and 7 and the inhibitor titer decreasing to 2 units/ml, but subsequently relapsed despite continued plasma exchange, with the inhibitor titer rising to a peak of 225 units/ml a few days prior to her death (Fig. 6, A and B). The addition of AD7 to an equal volume of each of the patient's plasma samples raised the protease levels to a maximum of 1.03 units/ml for

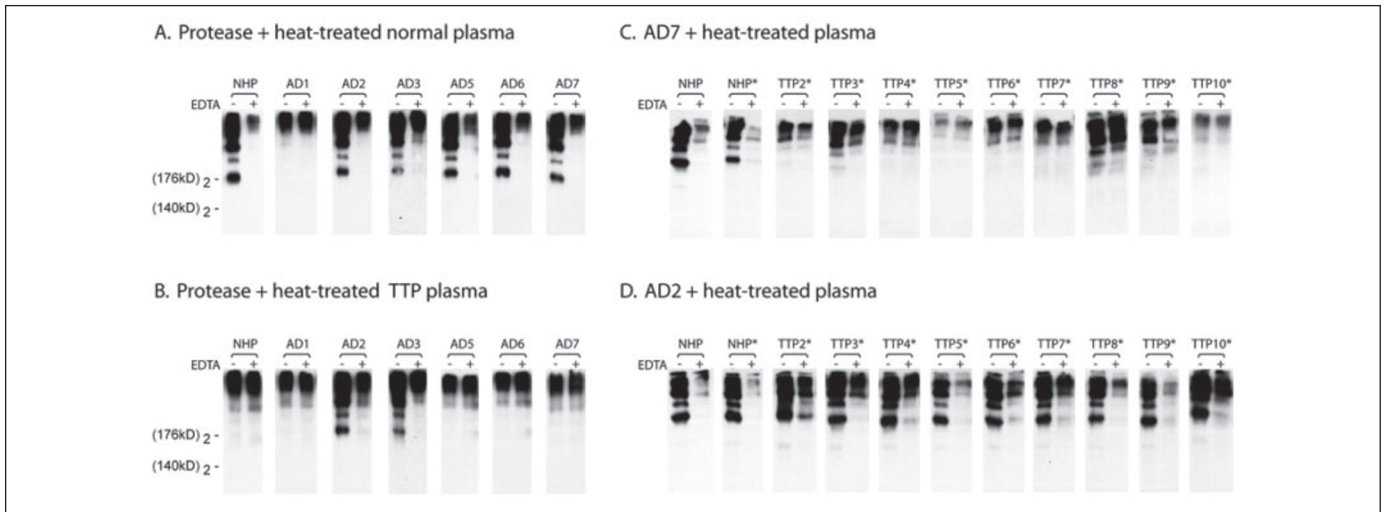


FIGURE 4. **Detection of VWF-cleaving activity in recombinant ADAMTS13 proteins and its suppression by TTP plasma.** A and B, immunoblots of SDS-PAGE depicting the VWF fragments ((176kD)₂, homodimer of the 176-kDa fragment; (140kD)₂, homodimer of the 140-kDa fragment, which is barely visible) generated after digestion of the VWF substrate by the ADAMTS13 activity in either normal human plasma (NHP) or the recombinant proteins. The catalytic activity of each recombinant protein was assayed after incubation with heated normal human plasma as a control (A) or with TTP plasma (B). Compared with heat-treated normal plasma in A, the heated TTP plasma in B suppressed the catalytic activity of AD5, AD6, and AD7, but not of AD2 or AD3. C and D, immunoblots of SDS-PAGE showing the VWF-cleaving activity in mixtures of AD7 (C) or AD2 (D) and heat-treated normal plasma (NHP*) or one of nine different TTP plasma samples. Each of the TTP plasma samples (TTP2–TTP10) suppressed the VWF-cleaving activity of AD7 but not AD2. To ensure the specificity of the assay, each digestion was conducted in the absence or presence of EDTA. EDTA suppressed the generation of VWF fragments observed in the absence of EDTA. Because of high concentrations, AD5–AD7 were diluted 1:20 for the analysis.

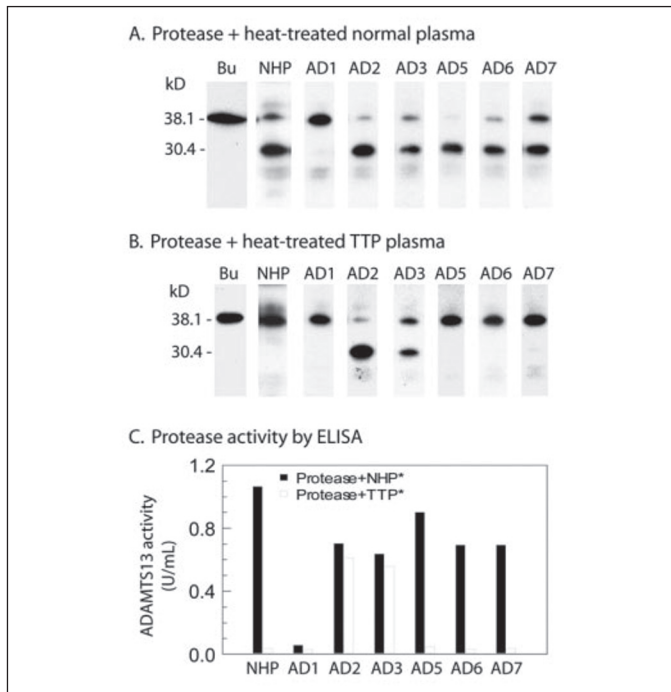


FIGURE 5. **Cleavage of GST-VWF73 (Asp¹⁵⁹⁶–Arg¹⁶⁶⁸)–His by recombinant ADAMTS13 proteins and its suppression by TTP plasma.** A, immunoblots of SDS-PAGE depicting the cleavage of GST-VWF73-His (38.1 kDa) to a 30.4-kDa band by the catalytic activity of normal human plasma (NHP) or of recombinant proteins AD2–AD7. Each protease was assayed after incubation with heat-treated normal human plasma as a control. Bu, Tris–NaCl buffer. B, after incubation with heat-treated TTP plasma, only AD2 and AD3 remained active in cleaving GST-VWF73-His. C, levels of GST-VWF73-His-cleaving activity as determined by ELISA. Compared with heat-treated normal human plasma (NHP*, filled bars), heat-treated TTP plasma (TTP*, open bars) suppressed the activity of AD5, AD6, and AD7 but not AD2 or AD3.

days 5 and 6, which was about one-tenth of the 10.5 units/ml obtained when the AD7 was added to heat-treated normal plasma. For day 8 and thereafter, the patient's plasma samples completely suppressed the activity of AD7 (Fig. 6C). In contrast, the addition of AD2 to the patient's

TABLE TWO

VWF-cleaving activity and interaction with TTP IgG of the ADAMTS13 variants

Numbers in the parentheses indicate the first and last amino acid residues of each construct. NA, not applicable.

Variant	Activity	Interaction with TTP IgG	
		Binding	Suppression
AD1 (1–310)	–	–	NA
AD2 (1–448)	+	–	–
AD3 (1–472)	+	–	–
AD4 (1–555)	+	–	–
AD5 (1–745)	+	+	+
AD6 (1–1238)	+	+	+
AD7 (1–1427)	+	+	+
AD8 (556–745)	–	–	NA
AD9 (449–745)	–	–	NA
AD10 (161–745)	–	+	NA
AD11 (161–555)	–	–	NA
AD12 (663–1238)	–	–	NA
AD13 (1016–1427)	–	–	NA

plasma samples raised the protease level to near the expected level (0.77 units/ml) throughout the course, irrespective of the levels of inhibitors (Fig. 6D). Similar mixing experiments with AD3 showed that none of the TTP plasma samples affected the protease activity of AD3.

DISCUSSION

The cloning of ADAMTS13 has opened new ways to improve the diagnosis and therapy of TTP. Because of technical complexity, the current functional assays for ADAMTS13 activity or inhibitory antibody levels have not been widely adopted in clinical laboratories. Furthermore, the presence of inhibitory antibodies complicates therapeutic approaches, and until now, no effective measures are available to rapidly suppress or remove the inhibitors of ADAMTS13. A better knowledge of the structure-function of ADAMTS13 and its interaction with the inhibitory antibodies of TTP patients may help overcome these obstacles.

ADAMTS13 Variants Resistant to TTP Autoantibodies

In this study, we investigated two groups of variant ADAMTS13 proteins. The first group, consisting of variants AD1–AD6, like full-length ADAMTS13 (AD7), contained the sequence of the native signal peptide, the propeptide, the metalloprotease, disintegrin, and TSP1 domains, but these proteins were truncated upstream from the carboxyl terminus. These proteins allowed us to study the VWF-cleaving activity as well as their binding with the antibody of TTP patients. The second group of variants AD8–AD13 lacked the amino terminus and was proteolytically inactive. These proteins helped define the segments of ADAMTS13 that were reactive with TTP IgG.

Comparison of the levels of recombinant proteins in the cell lysates and culture media revealed major differences in the levels of protein expression and secretion. Variants AD1, AD2, AD5, AD8, and AD13 were expressed and secreted more efficiently than the full-length AD7 protein, whereas AD4, AD10, and AD12 were secreted ineffectively. The reasons for these differences are not clear, and the level of expression could not be predicted from the primary sequences of the constructs.

The binding studies using either immunoprecipitation or ELISA revealed that variants AD5, AD6, and AD7, but not AD1–AD4, interacted with TTP IgG, suggesting that the sequence between residues 556 and 745 is essential for interaction with TTP IgG. Furthermore, our studies showed that AD10 (containing residues 161–745), but not AD8 (containing residues 556–745) or AD9 (containing residues 449–745), exhibited binding with TTP IgG, suggesting that the sequence from residue 449 or 556 to residue 745 alone is not sufficient for interaction with TTP IgG. One study has shown that the sequence between residues 449 and 688 is essential for ADAMTS13-TTP IgG binding (26). Other

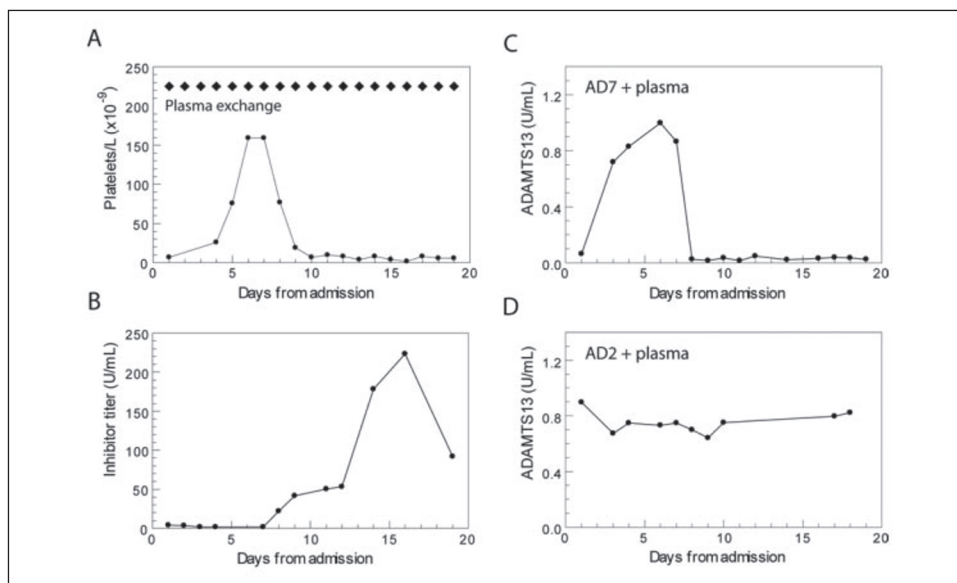
studies further report that proteins consisting of the sequence from residue 440 to 685 or from residue 556 to 685 are sufficient for recognition by TTP IgG (27, 28). Taken together, these studies consistently demonstrate that the spacer domain sequence is essential for recognition of ADAMTS13 protein by TTP IgG. However, the conclusions differ on whether recombinant proteins containing the cysteine-rich region and spacer domain are sufficient for interaction with TTP IgG. We speculate that the presence of the sequence of residues 686–745 in our AD8 or AD9 variants may have altered the reactivity of the spacer sequence with TTP IgG. Alternatively, these studies differ in the types of cells (bacteria, insect cells, or mammalian cells) used for expression of proteins and in the detection methodology (Western blotting *versus* immunoprecipitation or ELISA). The reason for the difference will need to be addressed in future studies.

One study using immunoblotting against bacterially expressed ADAMTS13 fragments suggests that the TTP IgG is reactive against multiple regions of ADAMTS13 (27). However, this epitopic promiscuity of TTP IgG has not been confirmed by another study, which demonstrated that although all TTP samples analyzed were reactive with the spacer fragment, only one of them exhibited binding with a non-spacer-containing sequence (28). Our studies of nine TTP patients did not detect binding between IgG and ADAMTS13 proteins lacking the spacer sequence, suggesting that few if any of the TTP IgGs are directed toward the TSP2-CUB region of the ADAMTS13. However, as variants AD8 and AD9 show, a mutant protein may contain a target epitope for TTP IgG but nevertheless fail to react with the antibody.

Analysis of proteolytic activity reveals that, compared with AD7, AD5 and AD6 are ~50–75% as active. This finding is similar to the results of a previous report (29). Previous studies further suggested that the spacer sequence was functionally essential for VWF-cleaving activity, because no protease activity was detected in the ADAMTS13 variants without the spacer sequence (26, 29). In contrast, our data showed that although AD2, AD3, and AD4 lacked the spacer sequence, they were proteolytically active. We consistently detected 0.4–0.8 units/ml protease activity in AD2. To understand the reasons for this discrepancy, we compared the specific activity (activity adjusted for molar concentrations) levels of the proteins and found that AD2 and AD3 were <1–2% as active as AD7. We obtained substantial VWF-cleaving activity levels in AD2 primarily because our constructs, particularly the AD2 variant were expressed much more efficiently than in previous studies. For example,

TABLE THREE				
Concentrations and activity levels of recombinant ADAMTS13 variants				
Variant	Secretion	Concentration (<i>versus</i> AD7)	Activity	Relative activity (<i>versus</i> AD7)
	%		<i>units/ml</i>	
AD1	60	17	0.0	0.000
AD2	48	25	0.77	0.003
AD3	12	0.4	0.06	0.016
AD5	38	4.3	17.0	0.41
AD6	19	2.4	16.8	0.73
AD7	2	1.0	9.5	1.00

FIGURE 6. Restoration of ADAMTS13 activity in TTP plasma with the ADAMTS13 variant protein AD2. A, the course of platelet counts in a TTP patient who relapsed through plasma exchanges and died on day 19. Each diamond represents one session of plasma exchange. B, the course of ADAMTS13 inhibitor titers in the same patient. C, the addition of AD7 to each of the patient's plasma samples raised the ADAMTS13 activity level on days 3–7 to a maximal value of 1.05 units/ml instead of the expected 10.5 units/ml. The plasma samples from day 8 and thereafter abolished the activity of AD7. D, the addition of variant AD2 (0.77 units/ml) to the patient's plasma samples raised the ADAMTS13 activity to the expected level, irrespective of the inhibitor titers.



the activity associated with a recombinant full-length ADAMTS13 was 1.4 units/ml in a previous study (29) but was nearly 10 units/ml in our study. Furthermore, the protein concentrations of AD2 were 25 times higher than the full-length AD7 in our study, whereas similarly truncated forms were only 2–3 times more concentrated than the full-length control in previous studies (26, 29). Thus, previous studies failed to detect the protease activity of ADAMTS13 mutants lacking the spacer or cysteine-rich/spacer sequences, because the proteins were analyzed at much lower concentrations.

Studies of another member of the ADAMTS13 family, ADAMTS4, have suggested that truncation of the protease at the cysteine-rich or spacer region decreases but does not abolish the proteolytic activity of the enzyme and that the catalytic domain by itself has very limited proteolytic activity (30). In agreement with these ADAMTS4 results, we found that successive deletion of the sequences of the CUB domains, the TSP1 2–8 motifs, the spacer domain, and the cysteine-rich sequence progressively lowered, but did not abolish, the VWF-cleaving activity of ADAMTS13 mutants. We did not detect protease activity with the AD1 variant that consists almost exclusively of the metalloprotease domain sequence. However, this does not preclude the possibility that protease activity in this variant might be detectable if tested at higher protein concentrations. Together, these results support the concept that the noncatalytic sequences modulate the protease activity of the ADAMTS proteases. Whether the truncated forms of ADAMTS13 also exhibit more promiscuous substrate specificity, as has been observed with ADAMTS4, remains to be explored. In SDS-PAGE analysis of VWF multimers after digestion with the truncated ADAMTS13 proteins, we did not detect novel proteolytic fragments.

One report has suggested that ADAMTS13 binds to VWF A3 domain, and that this binding may precede cleavage of the Tyr¹⁶⁰⁵–Met¹⁶⁰⁶ bond in the VWF A2 domain (31). In our study, the ADAMTS13 variants cleaved VWF multimers as well as a recombinant VWF fragment, GST-VWF73-His, that does not contain the A3 domain sequence. Thus, our data suggest that, at least *in vitro*, binding with the VWF A3 domain is not necessary for cleavage.

Since the ADAMTS13 mutants lacking the spacer or cysteine-rich/spacer sequences did not bind TTP IgG, it is not surprising that TTP IgG did not suppress the activity of AD2, AD3, or AD4. We tested the ADAMTS13 mutants against 10 random TTP plasma samples, all with inhibitor titers of at least 0.67 units/ml and found that although each abolished the VWF-cleaving activity of AD5, AD7, or normal plasma, none of them suppressed the protease activity of AD2. Furthermore, by investigating the serially collected samples of a severe case of TTP, we found that AD2 was unaffected by inhibitors as high as 225 units/ml.

The majority of TTP patients respond to plasma infusion or exchange therapy because they have low titers of ADAMTS13 inhibitors (<10 units/ml) (24). When the inhibitor titer is very high, as exemplified by the case in Fig. 6, plasma exchange or replacement with full-length protease would not be effective in raising the ADAMTS13 activity levels in the plasma. In this regard, ADAMTS13 variants that are not affected by TTP antibodies may have important advantages in therapeutic development of protease replacement. It will be important in future studies to define the *in vivo* clearance kinetics and the substrate specificity of the truncated forms of ADAMTS13, particularly when they are present at high concentrations.

In summary, our studies show that the noncatalytic sequence of ADAMTS13, particularly of the spacer domain, plays an important but not indispensable role in the VWF-cleaving activity of the protease. Because the spacer sequence is essential for recognition by TTP IgG, ADAMTS13 mutants lacking this sequence are proteolytically active but are not susceptible to suppression by TTP inhibitors. Compared

with the full-length protease, the truncated proteins, such as AD2, were secreted more efficiently, partially compensating for their lower proteolytic activity. Since clinical observations suggest that a level of 0.1–0.2 units/ml VWF-cleaving activity is sufficient to maintain hematological remission, variant forms of ADAMTS13 such as AD2 may be used effectively to bypass TTP inhibitors.

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