ADAMTS13, a metalloprotease, cleaves von Willebrand factor in plasma to generate smaller less thrombogenic fragments. The interaction of von Willebrand factor with specific ADAMTS13 domains was characterized with a binding assay employing von Willebrand factor immobilized on a plastic surface. ADAMTS13 binding was saturable and reversible. Equilibrium binding occurred within 2 h and the half-time for dissociation was ~4 h. Binding to von Willebrand factor was similar with either recombinant ADAMTS13 or normal plasma ADAMTS13; plasma from a patient that lacks ADAMTS13 activity showed no binding. The stoichiometry of binding was one ADAMTS13 per two von Willebrand factor monomers, and the $K_d$ was 14 nM. The ADAMTS13 metalloprotease and disintegrin domains did not bind VWF detectably. ADAMTS13 truncated after the first thrombospondin type 1 repeat bound VWF with a $K_d$ of 206 nM whereas ADAMTS13 truncated after the spacer domain had a $K_d$ of 23 nM, which is comparable to that of full-length ADAMTS13. Truncation after the eighth thrombospondin type 1 repeat reduced the binding affinity by ~3 fold and truncation after the seventh thrombospondin type 1 repeat in addition to the CUB domains increased the affinity for von Willebrand factor by ~2 fold. Therefore, the spacer domain is required for ADAMTS13 binding to von Willebrand factor. The first thrombospondin repeat also affects binding, and the C-terminal thrombospondin type 1 and CUB domains of ADAMTS13 may modulate this interaction.

Von Willebrand factor (VWF)$^1$ is a large multimeric protein that promotes hemostasis by tethering platelets at sites of vascular injury (1). The largest multimers of VWF are most active in platelet interactions, and thrombosis can result if large multimers accumulate as a result of ADAMTS13 deficiency. VWF is secreted into plasma as ultra-large multimers that are proteolyzed to smaller forms by the metalloprotease, ADAMTS13 (1-3). Deficiency of ADAMTS13 activity because of a congenital absence of the protein or because of the production of inhibitory anti-ADAMTS13 antibodies leads to thrombotic thrombocytopenic purpura (TTP) (4,5). TTP is characterized by uncontrolled microvascular thrombosis that especially affects the cerebral and renal circulation (6).

ADAMTS13 is a member of the ADAMTS family of metalloproteinases that are frequently involved in proteolysis of extracellular matrix proteins (7-11). The ADAMTS family is characterized by a modular domain structure consisting of a metalloprotease domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) repeat, a cysteine-rich region and a spacer domain (12). Most ADAMTS family members have a variable number of additional C-terminal TSP1 repeats. ADAMTS13 contains 7 additional TSP1 repeats and 2 CUB domains at its C-terminus and is the most divergent member of the family (13-15).

The CUB domains are unique to ADAMTS13 among the ADAMTS family members. CUB domains were first identified in the complement proteins C1r and C1s and have since been identified in many different proteins (16). In BMP-1 and cubilin, the CUB domains mediate interactions with procollagen and intrinsic factor-vitamin B$_{12}$ respectively (17,18) but in PDGF-C, the CUB domain inhibits the interaction with the PDGF-$\alpha$ receptor (19). The TSP1 repeats of ADAMTS family members have been shown to interact with the extracellular matrix (20) and to be...
important for substrate recognition and cleavage (21). The importance of the C-terminal TSP1 repeats and CUB domains in ADAMTS13 is suggested by their evolutionary conservation from Japanese pufferfish (Takifugu rubripes) through mice and humans (22) (J.E. Sadler, unpublished observations) although their functions remain unclear because ADAMTS13 truncated after the spacer domain retains full activity in cleaving VWF in vitro (23,24). Interestingly though, a peptide from the first CUB domain has been shown to inhibit cleavage of VWF by ADAMTS13 in a shear-dependent assay, suggesting that the C-terminal domains may interact with VWF (25).

To characterize the domains of ADAMTS13 that interact with VWF, we have investigated the binding of recombinant variants of ADAMTS13 to immobilized VWF. The $K_d$ for binding was comparable to the plasma concentrations of VWF and ADAMTS13, and the stoichiometry suggested that ADAMTS13 can bind to the majority of VWF subunits. Binding was strongly dependent on the spacer domain of ADAMTS13, and also appeared to be modulated by the most C-terminal TSP-1 and CUB domains.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-ADAMTS13 C-terminal antibody was made in rabbits against the predicted ADAMTS13 sequence EMQDPQSWKGEKEGT (amino acid residues 1414-1427) with a Cys residue added to the N-terminus (Pocono Rabbit Farm & Laboratory, Inc., Canadensis, PA). Rabbit polyclonal anti-human VWF was from Dako. Purified human VWF was a gift of C. Mazurier (CRTS, Lille, France).

Plasmid Constructs—A full length cDNA with C-terminal V5 epitope and (His)$_n$ tag encoding ADAMTS13 (23) was digested with HindIII and PmeI and ligated into the HindIII and EcoRV sites of pcDNA4/TO (Invitrogen). Plasmids encoding the C-terminal truncations of ADAMTS13 were prepared as described previously (23). These included constructs in which V5 epitope and (His)$_n$ tags were inserted after ADAMTS13 amino acid residues Q289, G385, E429, C555, or A685 cloned in pcDNA3.1D/V5-His-TOPO (Invitrogen); or after R1075 and A1191 cloned in pcDNA3.1/V5-His-TOPO (Invitrogen). All C-terminal truncation constructs were digested with HindIII and EcoRV and ligated into HindIII and EcoRV digested full-length ADAMTS13 in pcDNA4/TO.

Stable Transfections—T-REx™ 293 cells (Invitrogen) were cultured in Dulbecco’s modified Eagle’s medium containing 10% Tet-System Approved fetal bovine serum (Clontech, Palo Alto, CA), 2 mM L-glutamine, and 5 µg/ml blasticidin S. Cells were replated at approximately 90% confluence into 6-well dishes the day before transfection and they were transfected using 4 µg plasmid DNA and 60 µl Lipofectamine 2000 in 250 µl Opti-Mem (Invitrogen) according to the manufacturer’s directions. After 48 h, the cells were split 1:50 and 1:100 into Dulbecco’s modified Eagle’s medium containing 10% Tet-System Approved fetal bovine serum, 2 mM L-glutamine, 5 µg/ml blasticidin S and 300 µg/ml Zeocin™. Isolated colonies were picked after 14 days and propagated in the same medium.

Recombinant Protein Expression—For recombinant protein expression, stably-transfected cell lines were plated at 50% confluence and cultured in Opti-Mem containing 1 µg/ml tetracycline to induce expression from the tetracycline-inducible promoter of pcDNA 4/TO. Medium was collected after 3 days and 1µl/ml protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) and 1 mM phenylmethylsulfonyl fluoride were added. Medium was centrifuged at 600 × g for 10 min and the supernatant was concentrated at least twenty-fold by ultrafiltration using a Centriprep YM-30 (Millipore Corporation, Bedford, MA). The concentration of recombinant ADAMTS13 was measured using 7.5% SDS-PAGE and Western blotting with peroxidase conjugated-anti-V5 antibody and a chemiluminescent detection system (Amersham Biosciences). The luminograms were scanned, and the relative amount of protein detected was estimated by densitometry using NIH Image 1.62 (developed at National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image/). The concentration of V5-tagged ADAMTS13 constructs was determined by standardization with the V5-tagged Positope™ reference protein (Invitrogen) (23).

Binding Assay—Human VWF (7.5 µg/ml, 100 µl/well) in phosphate-buffered saline, pH 7.4, (PBS) was incubated in a 96 well Nunc-Immuno™
Plate MaxiSorp™ surface microtiter plate overnight at room temperature. Unbound VWF was removed and 200 µl of 2.5% bovine serum albumin in PBS containing 0.1% Tween 20 (Twen PBS) was added and incubated for 60 min. The wells were washed twice with Tween PBS and incubated with 100 µl of sample containing recombinant ADAMTS13 or plasma in 5 mM EDTA. Unbound protein was aspirated and the wells were washed with Tween PBS three times. For solubilization, 100 µl of 4X sample loading buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 712 mM β-mercaptoethanol and 1% bromophenol blue) was added. Solubilized protein was heated to 95 °C for 5 min and subjected to 5% SDS-PAGE, transferred by blotting to Immobilon P and detected with horseradish peroxidase-conjugated anti-V5 antibody (Invitrogen) and the ECL chemiluminescence detection system (Amersham Biosciences). Plasma ADAMTS13 was detected as above with the anti-ADAMTS13 C-terminal peptide antibody and peroxidase-conjugated swine anti-rabbit immunoglobulin antibody. For colorimetric assay, the wells were incubated with peroxidase conjugated anti-V5 antibody in Tween PBS containing 0.5% bovine serum albumin for 60 min and washed 3 times with Tween PBS. Color development was with 100 µl of 0.5 mg/ml o-phenylenediamine dihydrochloride as described above. The Ki was calculated using the equation IC50 = (1 + [immobilized VWF] / Kd)Ki using the Kd of 14 nM determined from the binding experiments described.

Steck-Wallach Plot—Data was plotted using the method of Steck and Wallach (26) according to the equation:

\[
\frac{\text{VWF}}{\text{Ki}} = \frac{\text{ADAMTS13}}{\text{n[ADAMTS13]}} + \frac{1}{\text{n}}
\]

where [ADAMTS13] = the concentration of free ADAMTS13, n = number of ADAMTS13 binding sites per VWF monomer, VWF = the number of VWF monomer subunits immobilized per assay well, ADAMTS13 = the number of ADAMTS13 bound, and Kd is the dissociation constant of ADAMTS13. The number of VWF monomers bound was determined by visualization of bound VWF using 5% SDS-PAGE, Western blotting with polyclonal peroxidase-conjugated anti-VWF (DAKO Corp., Carpinteria, CA) and the chemiluminescent ECL detection system. The luminograms were scanned and the relative amount of VWF was detected as described above. The absolute concentration was determined by standardization with known quantities of VWF. The number of ADAMTS13 molecules bound was determined as described above.

RESULTS

Time Course of ADAMTS13-VWF Association and Dissociation—The conformation of VWF is changed significantly by binding to hydrophobic (octadecyltrichlorosilane modified glass) or hydrophilic (mica) surfaces (27). Therefore, an assay system was developed to study the interactions of immobilized VWF and ADAMTS13. Cleavage of VWF in solution by ADAMTS13 requires denaturation of the VWF with guanidine, urea, or shear stress (2,3,28) but ADAMTS13 cleaved immobilized VWF in the
absence of denaturants (data not shown). Therefore, binding reactions were done in the presence of EDTA to inactivate ADAMTS13 (28). Bound ADAMTS13 was detected by solubilization of the microtiter well-bound protein to minimize dissociation that may occur during the antibody incubation step using the ELISA-based detection system.

Binding of recombinant ADAMTS13 was apparent after 1 minute of incubation and reached equilibrium by approximately 2 h (Fig. 1). Similar results were obtained if the reactions were done in the absence of EDTA although the extent of binding was less (data not shown).

The time course of dissociation was assessed in the presence of anti-VWF antibody to prevent rebinding of ADAMTS13. Bound ADAMTS13 decreased over time and dissociated ADAMTS13 increased reciprocally (Fig. 2A) with a half-time for dissociation of approximately 4 h (Fig. 2B). A relatively small fraction of bound ADAMTS13 (11-15%) was eluted in 60 min, making it feasible to measure binding by an ELISA-based colorimetric detection method in subsequent experiments.

**ADAMTS13 Binds Specifically to VWF**—ADAMTS13 did not bind significantly to immobilized bovine serum albumin demonstrating the specificity of ADAMTS13 for VWF over the more abundant plasma protein, albumin (Fig. 3A). ADAMTS13 binding to immobilized VWF was inhibited by rabbit polyclonal anti-VWF immunoglobulin and by immunoglobulin from TTP patients with auto-inhibitory antibodies against ADAMTS13, but not by non-immune rabbit immunoglobulin (Fig. 3A). Soluble VWF in PBS did not compete well with the immobilized VWF for ADAMTS13 binding, but soluble VWF did inhibit binding in reactions containing 1 M urea (Fig. 3B) indicating that urea may induce a change in VWF conformation similar to that caused by immobilization on a surface. The IC50 of the soluble VWF was 100 nM and the calculated \( K_i \) was 90 nM. Since the \( K_d \) of ADAMTS13 binding to VWF in 1 M urea solution is \( \sim 14 \) nM (data not shown), a conformational change in only a fraction of the urea-denatured VWF in solution may explain the disparity in the \( K_i \) and \( K_d \).

**ADAMTS13 from Normal Plasma Binds VWF**—Patients with TTP may lack plasma VWF cleaving protease activity because of a congenital absence of ADAMTS13 or because of the presence of anti-ADAMTS13 inhibitory antibodies. Plasma from normal blood donors or from a patient with TTP caused by inhibitory antibodies were used in the VWF binding assay. Bound ADAMTS13 increased when increasing concentrations of normal plasma were incubated with immobilized VWF, whereas bound ADAMTS13 was not detected when plasma from the patient with TTP was used (Fig. 4). Comparable binding was detected when either 100 ng of recombinant ADAMTS13 or 100 \( \mu \)l of fresh frozen plasma were used (data not shown) this result is consistent with a plasma concentration of 1 \( \mu \)g/ml ADAMTS13 that has been estimated previously (29). As was previously shown (Fig. 3B), the soluble VWF in plasma did not appear to inhibit the binding of ADAMTS13 to immobilized VWF. These results demonstrate that substantial binding of ADAMTS13 and VWF occurs readily in the complex milieu of plasma as well as in PBS.

**The Affinity and Stoichiometry of ADAMTS13/VWF Binding**—The binding of ADAMTS13 to a known amount of immobilized VWF was measured to determine the stoichiometry of binding, and a representative experiment is shown in Fig. 5. In this experiment, the stoichiometry of VWF subunits to ADAMTS13 was 2, and it ranged from 1 to 4 in other experiments. The \( K_d \) was determined to be 14 nM with a range from 9 to 16 nM. The \( K_d \) for binding of ADAMTS13 to immobilized VWF is comparable to the independently-determined \( K_m \) of 16 nM for the cleavage of VWF that was pretreated with guanidine-HCl (P. J. Anderson, unpublished results).

**Domains of ADAMTS13 Required for Binding VWF**—To define the domains of ADAMTS13 that interact with VWF, C-terminal truncations of ADAMTS13 were utilized in the binding assay. The constructs are named by the domain of ADAMTS13 with which they end (Fig. 6), and have a V5 epitope and (His)\(_6\) tag appended to the ADAMTS13 coding sequence. In previous studies of ADAMTS13 activity, C-terminal truncations that included at least the spacer domain were able to cleave VWF (23,24), suggesting that the spacer might interact directly with the substrate. When employed in direct binding assays, the Metalloprotease and Disintegrin constructs did not
bind VWF over the low background of nonspecific binding to bovine serum albumin (Fig. 7A). Constructs TSP1-1 and Cys-rich bound to VWF with relatively low affinity, and addition of the spacer domain to the Spacer construct restored binding further to approximately equal that of full-length ADAMTS13. These results support a major role for the spacer domain in the productive interaction of ADAMTS13 with VWF.

The binding activity of additional constructs with truncations toward the carboxyl-terminus suggest that more distal TSP1 and CUB domains also may affect binding to VWF. TSP1-8, which lacks the 2 C-terminal CUB domains, reproducibly had a slightly lower affinity for immobilized VWF compared to full-length ADAMTS13 (Fig. 7B). Removal of another TSP-1 repeat (TSP1-7 construct) however, reproducibly increased the binding affinity for VWF.

The $K_d$ values for the binding of selected C-terminal truncation constructs were calculated (Table I). The affinity of construct TSP1-1 for VWF is ~15-fold lower than that of full-length ADAMTS13 but substantially greater than that of the shorter Disintegrin and Metalloprotease constructs, which do not bind detectably. Removal of the 2 CUB domains (TSP1-8) reduces the affinity for VWF by ~3-fold, whereas removal of the 2 CUB domains and a C-terminal TSP1 repeat (TSP1-7) increases the affinity for VWF by ~2-fold, relative to full-length ADAMTS13. The $K_d$ of 23 nM for the Spacer construct is slightly higher than that of full-length ADAMTS13. Therefore, the interaction of the spacer domain with VWF is critical for binding, but binding also is affected by the proximal TSP1-1 domain, and possibly by the C-terminal TSP1-7, TSP1-8, and CUB domains.

**DISCUSSION**

ADAMTS13 binds specifically to immobilized VWF and with a binding affinity that is comparable to the independently determined $K_m$ for proteolytic activity. The $K_d$ is also near the estimated plasma concentration of ADAMTS13 of 8 nM (29), suggesting that modest changes in the plasma concentration of ADAMTS13 may significantly affect the hemostatic function of VWF. The stoichiometry of binding suggests that each ADAMTS13 molecule binds a monomer or dimer of VWF subunits (Fig. 5); several ADAMTS13 molecules might bind under sufficient fluid shear stress to each multimeric VWF molecule, which can contain as many as 40 subunits. Because soluble, non-denatured VWF does not compete for binding to immobilized VWF, binding of ADAMTS13 to VWF may not occur to an appreciable extent in the absence of fluid shear stress.

The inability of soluble VWF to prevent binding to immobilized VWF (Fig. 3B) suggests that the conformation of the VWF is changed by binding to the surface of the microtiter plate. Using atomic force microscopy, the structure of VWF was shown to be altered by adsorption on hydrophilic mica or on hydrophobic octadecyltrichlorosilane modified glass, and VWF had a much more extended structure on the hydrophilic surface (27). The microtiter plates used in the assay system consist of polystyrene coated with a MaxiSorp™ surface that promotes interactions with hydrophobic and hydrophilic portions of a protein, and have characteristics of both hydrophobic and hydrophilic surfaces. Although soluble VWF in PBS cannot compete for binding to immobilized VWF it can compete in the presence of 1 M urea (Fig. 3B). Therefore, surface binding of VWF may induce a change in conformation that has some similarity to the conformational change that is induced by urea; both enable binding of ADAMTS13. This conclusion is consistent with the observation that VWF and ADAMTS13 co-immunoprecipitate in the presence of 1 M urea but not in the absence of denaturants (30). Long “strings” of polymerized VWF bind to the endothelial cell surface after stimulation of VWF secretion with histamine, and this cell surface binding may also induce a conformational change in VWF that promotes interaction with ADAMTS13 (31).

ADAMTS13 binding to immobilized VWF reached equilibrium relatively slowly over ~2 h (Fig. 1). If the kinetics of binding are similar in vivo, equilibrium probably is not achieved in the circulation. The half-time for dissociation was ~4 h (Fig. 2). This slow dissociation time validated the use of an additional 60 min incubation step with a peroxidase-conjugated antibody for color development in an ELISA-style binding assay, which allows the rapid processing of many simultaneous reactions. Because binding of ADAMTS13 to VWF could be detected readily
using plasma samples from healthy controls but not from a patient with TTP (Fig. 4), this binding assay might be adapted for clinical determinations of ADAMTS13.

Using the ELISA method with C-terminal truncations of ADAMTS13, the contribution of specific structural domains to VWF binding could be estimated. The metalloprotease and disintegrin constructs do not detectably bind VWF as compared to other constructs (Fig. 7A). The TSP1-1 construct binds with a $K_d$ of $\sim$206 nM (Table 1), suggesting that the TSP-1 domain interacts with VWF, although the TSP-1 construct reportedly does not cleave full-length VWF (23,24). The apparent catalytic inactivity of the TSP1-1 construct suggests that additional critical binding sites are required for productive interaction with VWF. Alternatively, the assays employed to date may not be sufficiently sensitive to detect a low level of cleavage by this low affinity protease.

Addition of the cysteine-rich domain did not increase binding to VWF above that of TSP1-1 (Fig. 7A), but further addition of the spacer domain increased the affinity of binding $\sim$10-fold, nearly equal to the affinity of full-length ADAMTS13 (Table 1). The large contribution of the ADAMTS13 spacer to VWF binding is consistent with the observation that the spacer domain is required for ADAMTS13 cleavage of VWF in vitro (23,24). The central role of the spacer domain in productive substrate binding is supported by studies of antibodies to ADAMTS13 in TTP, which show that every patient with autoimmune TTP has had antibodies against the spacer domain (24,32,33).

C-terminal truncations after TSP1-7 or TSP1-8 gave additional interesting results. The nearly normal affinity of the Spacer construct for VWF might suggest that the C-terminal TSP-1 repeats and CUB domains are not needed for VWF interactions, and to date the presence or absence of these domains has had little effect on the proteolytic activity of ADAMTS13 truncation mutants (23,24). However, direct binding data (Fig. 7B) suggest that the C-terminal TSP1 domains and CUB domains may contribute to interactions with VWF. Compared to full-length ADAMTS13, there is a small but reproducible 2-fold decrease in the VWF affinity of TSP1-8, and a 3-fold increase in the affinity of TSP1-7 (Table 1). Two other studies also are consistent with a role for CUB domains in binding VWF. A synthetic peptide from the first CUB domain inhibited the binding of ADAMTS13-coated beads to VWF attached to the surface of endothelial cells (34). Also, ADAMTS13 and VWF can be co-immunoprecipitated in the presence of 1 M urea, and this interaction was blocked by a bacterially-expressed second CUB domain (30). The concordance among these various independent approaches suggests that distal TSP1 and CUB domains may be important for the binding and cleavage of VWF in vivo.

The accumulating data suggest that several structural domains of ADAMTS13 contribute to a complex, regulated interaction with its VWF substrate. The spacer domain clearly plays a major role, perhaps by binding to a specific feature of VWF that may be spatially removed from the cleaved Tyr$^{1605}$-Met$^{1606}$ bond, and this feature could becomes accessible under high fluid shear stress, upon binding to a surface, or in the presence of mild denaturants. Additional domains of ADAMTS13 may also participate, and direct binding data appear to implicate the proximal TSP1-1 domain, and the distal TSP1-7, TSP1-8, and CUB domains (Table 1). If several ADAMTS13 domains cooperate with the metalloprotease domain to recognize VWF, there may be a corresponding number of distinct sites on VWF with which these domains interact. Based on other studies that suggest a functional relationship between ADAMTS13 and specific domains of VWF, ADAMTS13 binding sites on VWF might reside in domain A1 (35), the C-terminal helix of domain A2 (36), and domain A3 (34). Further mutagenesis of ADAMTS13 and VWF should be useful to characterize such interactions.

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FOOTNOTES

1 The abbreviations used are TTP, thrombotic thrombocytopenic purpura; PBS, phosphate-buffered saline; TSP1, thrombospondin type 1; VWF, von Willebrand factor.

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FIGURE LEGENDS

FIG. 1. Time course of ADAMTS13 association with VWF. Recombinant ADAMTS13 was incubated with immobilized VWF for the indicated times in min. A, after removing the unbound ADAMTS13, the bound protein was solubilized and a fraction subjected to 5% SDS-PAGE and immunoblotting with anti-V5 antibody. B, the density of bands corresponding to bound ADAMTS13 was determined as described under Experimental Procedures and plotted as a function of incubation time for the western blot of panel A, which is representative of 4 independent experiments.

FIG. 2. Time course of ADAMTS13 dissociation from VWF. A, after equilibrium binding of ADAMTS13 to immobilized VWF, the unbound ADAMTS13 was removed and Tween PBS plus 1 µl anti-VWF was added to the well for the indicated times. The Tween PBS solution was removed and remaining bound proteins were solubilized. Samples of the soluble and bound fractions were subjected to 5% SDS-PAGE and immunoblotting with anti-V5 antibody to determine the fraction of bound and dissociated ADAMTS13. B, a plot of the percent of eluted ADAMTS13 versus time of dissociation. Densitometric analysis of the Western blot was performed with NIH Image 1.62. Error bars represent the SD for three experiments.

FIG. 3. ADAMTS13 binds specifically to VWF. A, recombinant ADAMTS13 was incubated in microtiter wells coated with bovine serum albumin (BSA) or VWF as indicated, in the presence (+) or absence (–) of purified IgG (50 µl of a 26 mg/ml solution) from patients with TTP who had autoantibodies against ADAMTS13, polyclonal anti-VWF (1 µl) or non-immune rabbit IgG (1 µl). After washing, bound ADAMTS13 was detected with peroxidase-conjugated anti-V5 antibody as described under “Experimental Procedures.” Results are expressed as a percentage of ADAMTS13 binding to immobilized VWF alone. Error bars represent the standard deviation from at least 3 experiments. B, soluble VWF in urea competes with immobilized VWF for the binding of ADAMTS13. Recombinant ADAMTS13 in 5 mM Tris-HCl, pH 8.0, 1 M urea (open squares, ☐) or in PBS (filled squares, ■) containing the indicated concentrations of soluble VWF was incubated in microtiter wells coated with VWF. After washing, bound ADAMTS13 was detected with peroxidase-conjugated anti-V5 antibody as
described under “Experimental Procedures.” Results are expressed as a percentage of binding obtained in the absence of added soluble VWF. Error bars represent the range of two experiments.

FIG. 4. ADAMTS13 in normal plasma binds immobilized VWF. The indicated volume of normal or TTP patient plasma was incubated with immobilized VWF for 3 h in a total reaction volume of 100 µl. Unbound plasma proteins were removed by washing. Bound proteins were solubilized and subjected to 5% SDS-PAGE and immunoblotting with anti-ADAMTS13 C-terminal peptide antibody and peroxidase-conjugated swine anti-rabbit immunoglobulin. Molecular mass standards in kilodaltons (kDa) are indicated on the left.

FIG. 5. Stoichiometry and affinity of ADAMTS13 binding to VWF. Binding reactions were performed with recombinant full-length ADAMTS13. A, bound ADAMTS13 was detected with peroxidase-conjugated anti-V5 antibody. B, bound ADAMTS13 and immobilized VWF were quantitated by solubilization, SDS-PAGE and immunoblotting with anti-V5 antibody or polyclonal anti-VWF antibody, respectively, to allow analysis by the method of Steck and Wallach (26). In this experiment, the value of $K_d$ was 12 nM and the stoichiometry was 2 VWF subunits per bound ADAMTS13.

FIG. 6. ADAMTS13 constructs. Secreted full-length recombinant ADAMTS13 (FL) consists of a metalloprotease domain, a disintegrin domain (Disintegrin), a thrombospondin type 1 repeat (TSP1 1), a cysteine-rich domain (Cys-rich), a spacer domain, seven additional thrombospondin type 1 repeats (TSP1 2-8), and two CUB domains. Truncation constructs encode ADAMTS13 through amino acid residue Gln289 (Metalloprotease), Gly385 (Disintegrin), Glu439 (TSP1-1), Cys555 (Cys-rich), Ala685 (Spacer), Arg1075 (TSP1-7), or Ala1191 (TSP1-8). All constructs contain a V5 epitope and (His)$_6$ tag at the C-terminus.

FIG. 7. Binding of ADAMTS13 constructs to immobilized VWF. Binding reactions were performed and bound recombinant ADAMTS13 proteins were detected with peroxidase-conjugated anti-V5 antibody. A, binding to immobilized VWF of Metalloprotease (open squares, □), Disintegrin (open diamonds, ◆), TSP1-1 (×), Cys-rich (filled triangles, ▲), Spacer (open circles, ○), and Full-length ADAMTS13 (filled squares, ■). Nonspecific binding is represented by the interaction of full-length ADAMTS13 with immobilized bovine serum albumin (+). B, binding to immobilized VWF of TSP1-7 (filled diamonds, ♦), TSP1-8 (open squares, □), and full-length ADAMTS13 (filled squares, ■).
Binding of ADAMTS13 variants to immobilized VWF

Binding of ADAMTS13 constructs to VWF was measured and values for $K_d$ were determined by fitting to a double-reciprocal version of the binding equation. In each case the linear regression coefficient was $> 0.99$. Table entries represent the average ± standard error of the mean for at least 3 independent experiments.

| ADAMTS13 Construct | $K_d$  |
|--------------------|--------|
|                    | nM     |
| Full length        | 14 ± 1.2 |
| TSP1-1             | 206 ± 38 |
| Spacer             | 23 ± 0.9 |
| TSP1-7             | 6.9 ± 1.5 |
| TSP1-8             | 45 ± 11  |
Figure 1

A

Time (min): 1 5 10 30 120 240 900

ADAMTS13

B

Bound (density)

0 1 2 3 4 5 6 7

0 200 400 600 800

Time (min)
Figure 2

A

Bound:

Eluted:

Time (min): 0 5 20 60 120 240 1320

B

ADAMTS13 Eluted (percent)

Time (min)
Figure 3

A

![Graph showing ADAMTS13 Bound (Percent) vs. Immobilized VWF levels.]

- Immobilized: BSA (0%), VWF (100%), VWF (80%), VWF (60%), VWF (40%), VWF (20%).
- Anti-ADAMTS13: 0%, 0%, 0%, 0%, 0%, 0%.
- Anti-VWF: 0%, 0%, 0%, 0%, 0%, 0%.
- Non-immune: 0%, 0%, 0%, 0%, 0%, 0%.

B

![Graph showing ADAMTS13 Bound (Percent) vs. VWF (nM) with PBS and Urea treatments.]

- PBS treatment: 100%, 80%, 60%, 40%, 20%.
- Urea treatment: 100%, 80%, 60%, 40%, 20%.

Legend:
- PBS
- Urea
| Plasma (µl) | Normal | TTP |
|------------|--------|-----|
| 250 kDa    | 50     | 100 |
| 150 kDa    |        |     |
Figure 5

A

B

ADAMTS13 Bound (A490 nm)

ADAMTS13 (nM)

VWF per ADAMTS13

1/ADAMTS13 (nM)
Figure 6
Figure 7

A

B
