Rearranging Exosites in Noncatalytic Domains Can Redirect the Substrate Specificity of ADAMTS Proteases*

Weiqiang Gao 1, Jian Zhu, Lisa A. Westfield, Elodee A. Tuley, Patricia J. Anderson, and J. Evan Sadler 2

From the Departments of Medicine and Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Background: ADAMTS metalloproteases are multidomain proteins with remarkable substrate specificity.

Results: Swapping noncatalytic domains between ADAMTS13 and ADAMTS5 causes reciprocal changes in the cleavage of their natural substrates.

Conclusion: ADAMTS exosites in noncatalytic domains are portable modifiers of proteolytic activity.

Significance: Shuffling and recombination of ADAMTS ancillary structural domains may be exploited to evolve or engineer new protease functions.

ADAMTS proteases typically employ some combination of ancillary C-terminal disintegrin-like, thrombospondin-1, cysteine-rich, and spacer domains to bind substrates and facilitate proteolysis by an N-terminal metalloprotease domain. We constructed chimeric proteases and substrates to examine the role of C-terminal domains of ADAMTS13 and ADAMTS5 in the recognition of their physiological cleavage sites in von Willebrand factor (VWF) and aggrecan, respectively. ADAMTS5 cleaves Glu373–Ala374 and Glu1480–Gly1481 bonds in bovine aggrecan but does not cleave VWF. Conversely, ADAMTS13 cleaves the Tyr1605–Met1606 bond of VWF, which is exposed by fluid shear stress but cannot cleave aggrecan. Replacing the thrombospondin-1/cysteine-rich/spacer domains of ADAMTS5 with those of ADAMTS13 conferred the ability to cleave the Glu1615–Ile1616 bond of VWF domain A2 in peptide substrates or VWF multimers that had been sheared; native (unsheared) VWF multimers were resistant. Thus, by recombining exosites, we engineered ADAMTS5 to cleave a new bond in VWF, preserving physiological regulation by fluid shear stress. The results demonstrate that noncatalytic thrombospondin-1/cysteine-rich/spacer domains are principal modifiers of substrate recognition and cleavage by both ADAMTS5 and ADAMTS13. Noncatalytic domains may perform similar functions in other ADAMTS family members.

The ADAMTS (a disintegrin-like and metalloprotease domain, with thrombospondin type-1 motif) superfamily contains 19 metalloproteases in a modular structure that includes a reprolysin-like metalloprotease domain (M), a disintegrin-like domain (D), a thrombospondin type 1 repeat (T), a Cys-rich domain (C), and a spacer domain (S), and a variable number of additional thrombospondin type 1 repeat and other domains. The ADAMTS superfamily also contains seven ADAMTS-like (ADAMTSL) proteins that lack M and D domains.

ADAMTS proteases, sometimes with assistance from ADAMTSL proteins, participate in many biological processes including procollagen processing, hemostasis, and extracellular matrix proteolysis relating to morphogenesis, angiogenesis, cancer, and osteoarthritis (1). For example, ADAMTS4 and ADAMTS5 degrade the cartilage proteoglycan aggrecan, which contributes to the development of arthritis. ADAMTS5 appears to be the major aggrecanase because it has higher aggrecanolytic activity than ADAMTS4, and genetic deletion of the ADAMTS5 catalytic domain protects mice from cartilage erosion in experimental models of osteoarthritis (2–4).

ADAMTS13 cleaves von Willebrand factor (VWF), which is required for normal platelet adhesion at sites of vascular injury. Interestingly, the susceptible peptide bond is buried in the native VWF A2 domain but is exposed when VWF is stretched as occurs in vivo within platelet-rich thrombi in flowing blood. Shear stress-induced VWF cleavage is an essential feedback inhibitory mechanism: congenital or acquired ADAMTS13 deficiency causes thrombotic thrombocytopenic purpura, which is characterized by life-threatening microvascular thrombosis (5–7).

Different ADAMTS proteases recognize very distinct substrates but employ similar mechanisms to establish strict substrate specificity: the metalloprotease domain determines cleavage site specificity, and C-terminal ancillary domains provide additional binding precision or localization (1). For example, ADAMTS4 and ADAMTS5 bind to the glycosaminoglycan chains of aggrecan and other extracellular matrix proteoglycans through the S domain (8) and C domain (9), respectively, and these interactions can profoundly affect substrate recognition. ADAMTS4 and ADAMTS5 both cleave aggrecan at several sites, including the Glu573–Ala574 bond in the interglobular domain (IGD) and the Glu1480–Gly1481 bond in the chondroitin sulfate-2 (CS-2) domain (bovine aggrecan numbering), and
deletion of the ADAMTS4 S domain (10) or the ADAMTS5 CS domains (9) markedly impairs cleavage at these sites. For ADAMTS13, optimal VWF cleavage depends on contacts between successive segments of the VWF A2 domain and corresponding binding sites in the proximal T, C, S, and distal thrombospondin type 1 repeat domains of ADAMTS13 (11–23).

The modular structure of ADAMTS active sites and exosites suggests that substrate specificity could be investigated and intentionally modified by reassorting metalloprotease and non-catalytic domains from different family members. The altered properties of chimeric proteases constructed from ADAMTS4 and ADAMTS5 support this concept (10). We have now created chimeric ADAMTS5 and ADAMTS13 proteases and have characterized their activity toward model aggrecan IGD and VWF substrates. The results confirm the modularity and portability of ADAMTS exosites and definitively that ADAMTS13 TCS domains specify shear stress-dependent cleavage of VWF by conferring that activity onto ADAMTS5 MD domains.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—An ADAMTS5 cDNA provided by Richard Leduc (Université de Sherbrooke, Sherbrooke, Canada) was used as the template for PCR amplification of a fragment encoding ADAMTS5 Met1–Thr863. The product was cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to yield plasmid pcDNA3.1/MDTCS5. Chimeric enzymes (see Fig. 1A) were constructed by overlapping PCR using pcDNA3.1/MDTCS13 (24) or pcDNA3.1/MDTCS5 as the template. The PCR products, which included adjacent polylinker sequences from pcDNA3.1/V5-His-TOPO, were digested with HindIII and PmeI and ligated into the HindIII and EcoRV sites of pcDNA4/TO (Invitrogen).

Plasmid pGST-VWF73 was described previously. Plasmid pGST-IGD was constructed similarly in *Schistosoma japonicum* GST fusion expression vector pGEX-6P-1 (GE Healthcare) using a QuickChange II site-directed mutagenesis kit (Stratagene) with human aggrecan cDNA (MGC-26414, ATCC) as the template and the following primers: for VWF/IGD 5′-ATGGCGGCT-3′ (forward) and 5′-CTTC-3′ (reverse); for IGD/VWF 5′-CTCAGTGATGGTGATGGTGATGCCCTCCTGGCAAATGCGGCT-3′ (forward), and 5′-CTTCAGTGATGGTGATGGTGATGCCCTCCTGGCAAATGCGGCT-3′ (reverse). Megaprimer’s chimeric substrate construction (25) were amplified using pGST-IGD as the template and the following primers: for VWF/IGD 5′-CGGAATTCTCCTCCCTATGAGATC/GAACCAGGGAGCCTCC-3′ (forward), and 5′-CTCAGTGATGGTGATGGTGATGGTGATGGTGATGCCCTCCTGGCAAATGCGGCT-3′ (reverse); for IGD/VWF 5′-TCTGTTCCAGGGGCCCTTGACAGACCTTTTTG-3′ (forward), 5′-CTCAGTGATGGTGATGGTGATGGTGATGGTGATGCCCTCCTGGCAAATGCGGCT-3′ (reverse). The desired chimeric sequences were amplified using purified PCR products as megaprimers and pGST-VWF73 as the template. Digestion with DpnI was increased to 3 h to completely remove methylated template DNA. Products were electroporated into *Escherichia coli* XL-Blue cells, and clones were selected based on restriction digestion, PCR, and DNA sequencing.

**Recombinant Proteases**—T-Rex 293 cells (Invitrogen) were transfected with plasmids encoding ADAMTS variants (10 μg for transient expression or 1 μg for stable expression) using Lipofectamine 2000 (Invitrogen). Transiently transfected cells were induced to express proteases in Freestyle serum-free medium (Invitrogen) with 1 μg/ml tetracycline. Stable cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% tetracycline-approver fetal bovine serum (Clontech or Invitrogen), 300 μg/ml zeocin, 5 μg/ml blasticidin, 2 mM glutamine, 5 units/ml penicillin, and 5 μg/ml streptomycin. Protein expression was initiated in 70–80% confluent roller bottles with 1 μg/ml tetracycline in Freestyle serum-free medium. This concentration of tetracycline does not inhibit ADAMTS13. Heparin (100 μg/ml) was added to the medium for cell lines expressing MDT13/CS5, MD13/TCS5, or MDTCS5. Conditioned media were centrifuged, and filtered, and serine protease inhibitors were added (0.1 μM D-Phe-Pro-Arg-Ch_{2}Cl (FPR-CK), 0.1 μM Phe-Phe-Arg-Ch_{2}Cl (FRR-CK), and 144 μM phenylmethylsulfonyl fluoride).

ADAMTS protein concentrations were determined as described (11) by SDS-PAGE with V5-tagged Positope reference protein standards (Invitrogen), Western blotting on PVDF membranes with anti-V5 antibody and peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution, A3673; Sigma), and chemiluminescence detection (ECL Plus; GE Healthcare). Signals were collected and analyzed with a fluorescence imaging system (Typhoon Trio; GE Healthcare) and ImageQuant TL software (GE Healthcare). The concentration of ADAMTS proteases in conditioned medium typically was 12–36 nm (1–3 μg/ml). The media were concentrated 5–10-fold by ultrafiltration on YM30 membranes (Millipore, Inc.) and dialyzed into an appropriate reaction buffer. Proteases were aliquoted and stored at −30 °C until used without further purification. When assayed with substrate FRETS-VWF73 (26), the activity of 0.6 nm MDTCS13 was equivalent to that of 0.6 nm ADAMTS13 in standard pooled normal plasma.

**Substrates**—Substrate gst-VWF73 (37.5 kDa), gst-VWF/IGD (39 kDa), gst-IGD/VWF (42 kDa), and gst-IGD (44 kDa) were expressed and purified from the soluble fraction of *E. coli* lysates as described previously for gst-VWF73 (14). After dialysis against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, the protein concentration was determined with a BCA protein assay kit (Pierce) and a BSA standard.

**Substrate Cleavage by Recombinant ADAMTS Variants**—The reactions included 40 μl of reaction buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM CaCl_{2}, 0.1 μM ZnCl_{2}, 0.9 μl of BSA (5 mg/ml), 0.1 μl of (2-aminoethyl)benzenesulfonyl fluoride (40 mM), 0.1 μg of GST-peptide substrate, and 22.5 mM protease to make a total volume of 45 μl. The reactions were incubated at 37 °C and stopped by adding an equal volume of 2× SDS sample buffer containing 20 mM EDTA. Samples (10 μl) were analyzed by SDS-PAGE on 10–20% gradient gels (Invitrogen), electrotransferred onto PVDF membranes, and incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated rabbit anti-GST antibody (GE Healthcare), and chemiluminescence detection (ECL Plus; GE Healthcare).

To assess catalytic efficiency (k_{cat}/K_{m}), reactions containing 3–7 nm substrate, 0.5 or 1 mM protease, and 0.05% Tween 20...
were incubated at 37 °C for different times (t) and stopped with an equal volume of 50 mM EDTA, and products were analyzed by sandwich ELISA as described (16). Briefly, reactions were adsorbed onto microtiter plates coated with anti-GST antibodies (Pierce), and uncleaved substrates were detected with peroxidase-conjugated anti-His antibodies (Invitrogen). MDTCS13 cleaves GST-VWF73 with $K_m = 800$ nM (14), and bovine aggrecan (principally ADAMTS5) cleaves a 40-residue bovine IGD peptide substrate with $K_m \approx 480$ µM (27). Therefore, in all reactions the enzyme concentration ($E$) and initial substrate concentration ($S_0$) are much lower than the $K_m$, and the time courses of product ($P$) generation were analyzed to obtain $k_{cat}/K_m$ (corresponding to the initial rate) by fitting to the following integrated Michaelis-Menten equation.

$$P/S_0 = (1 - e^{-t/k_{cat}/K_m})$$

(Cleavage Site Characterization)—GST-peptide substrates (5 µg) were incubated with ADAMTS protease (10 nM) in reaction buffer at 37 °C. After 0.5 h, substrate cleavage was confirmed by SDS-PAGE and Western blotting using peroxidase-conjugated anti-His tag antibody (Tetra-His; Qiagen). After incubation for 5 h, the products were separated by SDS-PAGE on a 10–20% gradient gel, transferred onto a PVDF membrane (0.2 µm; Invitrogen), and stained with SimplyBlue SafeStain (Invitrogen). C-terminal product bands (8.8–11 kDa) were excised from the membrane and sequenced by automated Edman degradation (W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University).

Aggrecan Cleavage—ADAMTS Variants were incubated with 750 nM bovine aggrecan (Sigma-Aldrich) in reaction buffer at 37 °C, and the reactions were terminated by the addition of an equal volume of 20 mM EDTA. Digestion products were deglycosylated overnight with chondroitinase ABC (0.01 unit/10 µg aggrecan) (Sigma-Aldrich) and keratanase (0.01 unit/10 µg aggrecan) (Sigma-Aldrich). The aggrecan core protein was preincubated overnight with chondroitinase ABC (0.01 unit/10 µg aggrecan) (Sigma-Aldrich) and the time courses of product ($P$) generation were analyzed to obtain $k_{cat}/K_m$ (corresponding to the initial rate) by fitting to the following integrated Michaelis-Menten equation.
observed between intracellular and secreted MD13/CS5, MD13/CS5, or MDTCS13 (Fig. 1B).

A faint 54-kDa species was detected with anti-V5 antibody in some recombinant proteases that may be a C-terminal proteolytic fragment (Fig. 1B). If so, the size of the fragment is consistent with cleavage in the metalloprotease domain.

**ADAMTS Substrates**—Chimeric GST-peptide substrates based on the sequences of human VWF and aggrecan (Fig. 1C) were expressed in *E. coli* and purified to homogeneity (Fig. 1D). Substrate gst-VWF73 corresponds to the 73-residue Asp1596–Arg1668 segment of VWF, which is the smallest VWF fragment that is cleaved efficiently by ADAMTS13 (12). ADAMTS13 D, T, C, and S domains interact with successive segments of this substrate between Asp1596 and Arg1668, and these cooperative interactions accelerate the cleavage of the Tyr1605–Met1606 bond by several orders of magnitude (16, 17, 19, 20).

A substrate gst-IGD contains the Thr331–Gly459 IGD segment of human aggrecan. A similar GST-peptide substrate containing aggrecan Tyr330–Gly457 and a C-terminal FLAG tag (34) is previously reported to be cleaved efficiently by bovine aggrecanase (27).

**Substrate Specificity of ADAMTS Variants**—Recombinant proteases in conditioned medium were concentrated by ultrafiltration, dialyzed against reaction buffer, and used without further purification. The activity of ADAMTS proteases toward chimeric substrates was assessed by a semiquantitative Western blot assay method. As expected (16), proteases containing the MD domains of ADAMTS13, MDTCS13, MD13/CS5, and MD13/CS5, cleaved gst-VWF73 and gst-VWF/IGD to a detectable extent (Fig. 2A), even after increasing the time of digestion from 2 to 24 h or increasing the enzyme concentration 10-fold (Fig. 2B).

Proteases containing the MD domains of ADAMTS5, MDTCS5 and MD5/TCS5, cleaved gst-IGD and gst-IGD/VWF to generate a 32-kDa product (Fig. 2A). To establish the
site of cleavage, MD5/TCS13 was incubated with gst-IGD and gst-IGD/VWF, and C-terminal cleavage products were isolated and sequenced. The N-terminal sequence obtained was ARGS-VILTVKP in each case, indicating that MD5/TCS13 cleaves both substrates at the Glu373–Ala374 bond (Fig. 3).

MDTCS5 could not cleave substrates gst-VWF73 and gst-VWF/IGD, but MD5/TCS13 was able to cleave them (Fig. 2), and the mobility of the N-terminal product on SDS-PAGE was similar to that generated by MDTCS13. Automated Edman degradation of the C-terminal product gave the sequence IKRLPGDI (Fig. 3 B), indicating that MD5/TCS13 cleaves the Glu1615–Ile1616 bond of gst-VWF73 or gst-VWF/IGD, 10 residues C-terminal of the Tyr 1605–Met1606 bond cleaved by MDTCS13 (16). Prolonged incubation for 24 h or with 10-fold more MD5/TCS13 resulted in complete cleavage of gst-VWF73 but partial cleavage of gst-VWF/IGD (Fig. 2 B). Thus, TCS13 domains confer on MD5 the ability to cleave substrates based on the VWF A2 domain, and the chimeric MD5/TCS13 protease prefers gst-VWF73, which contains sequences that interact with TCS13 domains (12, 14, 16, 17).

**Catalytic Efficiency of Substrate Cleavage by ADAMTS Variants**—The time course of substrate cleavage (Fig. 4) was analyzed under conditions such that $E \ll K_m$ and $S_0 \ll K_m$. Fitting to the integrated Michaelis-Menten equation yields values for $k_{cat}/K_m$, a measure of catalytic efficiency (Table 1).

Substrate gst-VWF/IGD lacks the exosite-binding sequences of gst-VWF73 that are important for VWF recognition (12, 16, 17, 19, 20), and MDTCS13 cleaved gst-VWF73 ~5-fold more rapidly than gst-VWF/IGD as expected (Table 1). MDT13/CS5 cleaved both substrates more slowly than MDTCS13 but also preferred gst-VWF73 over gst-VWF/IGD as expected (Table 1). The Glu373–Ala374 bond in gst-IGD/VWF and gst-IGD is cleaved by MD5/TCS13. Amino acid residues identified by Edman degradation are underlined. A caret indicates the boundary between sequences exchanged in substrates gst-VWF/IGD and gst-IGD/VWF.

than MDT13/CS5 and also discriminated less effectively between gst-VWF73 and gst-VWF/IGD; the difference in cleavage rate was ~4-fold for MD13/TCS5 compared with
The indi-
cated GST-substrates were incubated at 37 °C with MDTCS13 (red squares),
MDT13/CS5 (orange diamonds), MD13/TCS5 (yellow circles), MDS/TCS13 (green triangles), or MD5/TCS5 (blue triangles). The concentration of protease
was 1 nM except for the reaction of MDTCS13 (0.5 nM) and VWF73. Cleavage
products were quantitated by ELISA, and values for
were determined
by fitting to the integrated Michaelis-Menten equation (Table 1). The data
points represent the mean values for at least two independent experiments.

10-fold for MDTCS13/CS5 (Table 1). Thus, replacement of the
ADAMTS13 T domain made substrate recognition less
dependent on substrate sequences after VWF Ile1616.

Replacing the distal domains of MDTCS5 gave complementa-
ry results but with interesting differences. MDTCS5 cleaved
substrate gst-IGD with efficiency similar to that with which
MDTCS13 cleaved gst-VWF73. MDTCS5 also cleaved gst-IGD
and gst-IGD/VWF with similar efficiency, indicating that the C-terminal 65 residues of the IGD domain, Glu373–Gly381,
contribute little to substrate recognition by distal domains of
MDTCS5. MD5/TCS13 cleaved gst-IGD/VWF ~2.2-fold more
rapidly than gst-IGD (Table 1), indicating that TCS13 domains
confer a modest preference for substrates containing their natural
binding partners in the Lys1617–Arg1668 segment of VWF.

More strikingly, MD5/TCS13 acquired the ability to cleave
gst-VWF73 with surprising efficiency. MDTCS5 was inactive
toward gst-VWF73, but MD5/TCS13 cleaved gst-VWF73 at
nearly one-third the rate of MDTCS13 (Table 1). In addition,
MD5/TCS13 cleaved gst-VWF73 ~10-fold faster than
gst-IGD, indicating that efficient gst-VWF73 recognition
was mediated by interactions between distal segments of
gst-VWF73 and exosites in TCS13 domains.

Aggrecanase Activity of Recombinant ADAMTS Enzymes—
The glycosaminoglycan chains of aggrecan bind ADAMTS5
and influence cleavage by this protease. However, gst-IGD
and gst-IGD/VWF substrates (Fig. 1) are not glycosylated and
cannot be used to evaluate this relationship. To assess the contribu-
tion of glycosylation to substrate recognition, ADAMTS
variants were incubated with bovine aggrecan, and specific neo-
epitopes produced by cleavage were detected by Western blot-
ting (Fig. 5).

ADAMTS13 has not been shown to cleave after Glu residues
in any substrate, and proteases MDTCS13, MD13/CS5, and
MD13/TCS5 did not generate any products corresponding to
cleavage at Glu373–Ala374 (Fig. 5A) or Glu373–Gly381 (Fig.
5C). However, both MDTCS5 and MD5/TCS13 cleaved aggrecan
at Glu373–Ala374 in the IGD (Fig. 5A), and the time course
of cleavage indicated they have approximately equal activity
toward this site (Fig. 5B). In contrast to their similar IGD cleav-
ing activity, MDTCS5 cleaved the Glu1480–Gly1481 site in the
aggrecan CS-2 domain, but MD5/TCS13 did not (Fig. 5C).

Deletion mutagenesis of ADAMTS5 indicates that truncated
MD5 cannot cleave either the Glu373–Ala374 or Glu1480–
Gly1481 site (9). Appending TCS13 domains in MD5/TCS13
restored cleavage only at Glu373–Ala374 (Fig. 5), indicating that
TCS13 exosites discriminate between the aggrecan IGD and
CS-2 domains.

Shear-dependent Cleavage of VWF—MD5/TCS13 acquired
the ability to cleave the Glu1615–Ile1616 bond of gst-VWF73,
and therefore multimeric VWF was also evaluated as a sub-
strate (Fig. 6). Plasma VWF and ADAMTS proteases were incu-
bated under static conditions or sheared by vortexing for 200 s.
The reactions were analyzed by SDS-PAGE and Western blot-
ing to detect VWF cleavage products (21, 29). As a negative
control, the reactions were supplemented with EDTA before
vortexing. As reported previously (21, 29), plasma VWF was
resistant to cleavage by MDTCS13 under static conditions but
was cleaved rapidly when the reaction was subjected to fluid
shear stress. VWF was resistant to cleavage by MDTCS5, with
or without shear stress. However, the results for MD5/TCS13
were similar to those for MDTCS13. VWF was not cleaved by
MD5/TCS13 under static conditions but was cleaved under
fluid shear stress. Thus, recombining exosites enabled MD5/
TCS13 to perform shear-dependent cleavage of VWF
multimers.

DISCUSSION

The noncatalytic domains that characterize the ADAMTS
protease family mediate several distinct functions in different
family members, including substrate recognition, tissue local-
ization, and angiogenesis (1). We have used a domain substitu-
tion approach to investigate the role of noncatalytic domains
in ADAMTS5 and ADAMTS13. In addition we prepared chime-
ric model substrates in which cleavage sites and ancillary bind-
ing sites were exchanged between VWF domain A2 and aggrecan
IGD. The results show that the catalytic center and
noncatalytic domains can cooperate to determine substrate
specificity, but the importance of each interaction varies con-
siderably for different enzyme-substrate combinations.

MDTCS5 cleaved gst-IGD efficiently as expected but also
cleaved gst-IGD/VWF at essentially the same rate. Further-
more, MD5/TCS13 cleaved gst-IGD rapidly despite the
absence of distal ADAMTS5 domains. These results suggest
that the C-terminal segment of the nonglycosylated IGD poly-
peptide does not interact functionally with TCS5 exosites.
In addition, MDTCS5 and MD5/TCS13 both cleaved the Glu373–
Ala374 bond of native bovine aggrecan with similar efficiency,
indicating that TCS5 exosites that interact with glycosaminogly-
cans (9) may not contribute to cleavage of the aggrecan IGD.

Although MD5/TCS13 cleaved the aggrecan IGD efficiently,
it could not cleave the Glu1480–Gly1481 bond in the aggrecan
CS-2 domain. However, studies of ADAMTS5/ADAMTS4 chime-
ric proteases showed that MD5/TCS4 cleaved both IGD and
CS-2 sites normally (10). Thus, when combined with MD5,
TCS4 can functionally replace TCS5 for both IGD and CS2
cleavage, whereas MD5/TCS13 only preserves IGD cleavage.
Exosites and ADAMTS Substrate Specificity

Table 1: Efficiency of substrate cleavage

| Enzyme          | VWF73 | VWF/IGD | IGD/IGD | IGD |
|-----------------|-------|---------|---------|-----|
| MDTCS13         | 49 ± 5| 11 ± 1  | -       | -   |
| MDT13/CS5       | 26 ± 2| 2.3 ± 0.3| -       | -   |
| MD13/TCS13      | 6.0 ± 0.9| 1.5 ± 0.3| -       | -   |
| MD5/TCS13       | 15 ± 1| 1.5 ± 0.4| 144 ± 16| 65 ± 5|
| MDTCS5          | -     | -       | 81 ± 6  | 83 ± 5|

These results are consistent with a model proposed by Fushimi et al. (10) in which cleavage of the Glu373–Ala374 site in the aggrecan IGD was assessed by incubating bovine aggrecan (750 nM) with the indicated enzyme (1 nM) in reaction buffer at 37 °C for 4 h. The data shown are representative of at least three independent experiments.

FIGURE 5. Cleavage of aggrecan by ADAMTS proteases. A and B, cleavage at Glu373–Ala374 in the aggrecan IGD was assessed by incubating bovine aggrecan (750 nM) with the indicated enzyme (1 nM) in reaction buffer at 37 °C for 4 h (A) or the indicated time (B). The reactions were terminated with 50 mM EDTA, and samples were deglycosylated and analyzed by SDS-PAGE and Western blotting with antibody BC-3, which recognizes N-terminal 374ARGS epitope antibodies. This result probably reflects the extraordinary strict specificity of ADAMTS13 for the sequence Leu-Xaa-Tyr-Met (35, 36), which occurs once in VWF and not at all in aggrecan.

Dramatically altered the specificity of MD5, enabling it to cleave an otherwise resistant substrate, and cleavage depended on TCS13 exosite interactions with gst-VWF73 that cannot occur for gst-VWF/IGD. These exosite-substrate interactions are consistent with loss of function phenotypes induced by engineered deletion and missense mutations in ADAMTS13 and VWF (11–17, 19, 22, 23). The gain of function produced by transferring functional ADAMTS13 exosites onto ADAMTS5, in construct MD5/TCS13, shows definitively that TCS13 domains are critical for recognizing and cleaving VWF because they bind to a C-terminal segment of the A2 domain.

Although the ADAMTS5 metalloprotease domain could be induced to cleave VWF in construct MD5/TCS13, none of the tested proteases that contained an ADAMTS13 active site was able to cleave aggrecan IGD sequences or native aggrecan core protein, at least at the sites recognized by aggrecanase neoepitope antibodies. This result probably reflects the extraordinary strict specificity of ADAMTS13 for the sequence Leu-Xaa-Tyr-Met (35, 36), which occurs once in VWF and not at all in aggrecan.

The physiological cleavage of VWF by ADAMTS13 is regulated by fluid shear stress, which unfolds the A2 domain and exposes the buried scissile bond. MDTCS5 did not cleave plasma VWF detectably, but chimeric MD5/TCS13 acquired the ability to cleave VWF in a shear-dependent manner, most likely at the Glu1615–Ile1616 bond that is cleaved in gst-VWF73. The A2 domain crystal structure, Glu1615–Ile1616 is located on the surface in an extended segment referred to as the o4-less loop (37). The side chain of Glu1615 projects into solvent, but Ile1616 is buried. Exposure of this bond by shear-induced unfolding of VWF was not sufficient to render it sensitive to MDTCS5. However, exosites conferred by TCS13 domains
enabled MD5/TCS13 to recognize and cleave VWF with remarkable efficiency and with regulation by fluid shear stress. The Glu1615–Ile1616 selected by MD5/TCS13 is one of six Glu-Xaa bonds in gst-VWF73, and the predilection for this bond reflects the specificity of the ADAMTS5 metalloprotease domain, as well as geometric constraints on positioning a substrate between the active site and exosites. ADAMTS5 prefers certain Glu-Gly, Glu-Leu, and Glu-Ala bonds in aggrecan (4), but little is known about its specificity for extended sequences adjacent to these sites. It seems likely that modifying the scissile bond environment to better match the specificity of ADAMTS5 would further enhance the shear-dependent cleavage of VWF by MD5/TCS13.

The evolution of ADAMTS proteases has involved many instances of domain duplication and shuffling (38). These proteins have diverged considerably—ADAMTS5 and ADAMTS13 are just 29% identical over the domains they share—and the reshuffling of domains in vitro is a powerful strategy to investigate structure-function relationships. Our studies and others (10, 39, 40) indicate that ADAMTS noncatalytic domains often are portable, easily transferred between family members with retention of structural integrity. In addition, their exosite functions tend to be modular, corresponding to one or a few domains. Finally, the lack of stringent distance constraints allows new combinations of exosites and active sites to manifest new proteolytic activities, which could have practical applications. For example, MD5/TCS13 might be able to restore regulated proteolysis of VWF in patients with thrombotic thrombocytopenic purpura caused by autoantibodies against the MD domains of ADAMTS13.

Noncatalytic domains strongly determine the specificity of ADAMTS5 and ADAMTS13, supporting the principle that ADAMTS proteases require their noncatalytic domains to recognize substrates and localize their proteolytic activity (1). A corollary might be that targeting of noncatalytic domains should inactivate ADAMTS proteases in vivo. In a way, this concept is validated by the observation that autoantibodies against the ADAMTS13 spacer domain can inhibit protease activity enough to cause thrombotic thrombocytopenic purpura (5, 6). This proof of principle experiment of nature suggests that intentionally targeting the exosites of other ADAMTS proteases may be therapeutically useful.

Acknowledgments—We thank Dr. Richard Leduc (Université de Sherbrooke) for an ADAMTS5 cDNA, and Dr. Hideaki Nagase (Imperial College London) for anti-GELE480 cleavage site antibody.

REFERENCES
1. Apte, S. S. (2009) A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily. Functions and mechanisms. J. Biol. Chem. 284, 31493–31497
2. Glasson, S. S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H. L., Flannery, C. R., Peluso, D., Kanki, K., Yang, Z., Majumdar, M. K., and Morris, E. A. (2005) Deletion of active ADAMTS5 prevents carilage degradation in a murine model of osteoarthritis. Nature 434, 644 – 648
3. Stanton, H., Rogerson, F. M., East, C. J., Golub, S. B., Lawlor, K. E., Meeker, C. T., Little, C. B., Last, K., Farmer, P. J., Campbell, I. K., Foureie, A. M., and Fosang, A. J. (2005) ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. Nature 434, 648 – 652
4. Fosang, A. J., Rogerson, F. M., East, C. J., and Stanton, H. (2008) ADAMTS-5. The story so far. Eur. Cell Mater. 15, 11 – 26
5. Furlan, M., Robles, R., Galbusera, M., Remuzzi, G., Kyrie, P. A., Brenner, B., Krause, M., Scharrer, I., Aumann, V., Mittler, U., Solenthaler, M., and Lämmlle, B. (1998) von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. N. Engl. J. Med. 339, 1578 – 1584
6. Tsai, H. M., and Lian, E. C. (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. N. Engl. J. Med. 339, 1585 – 1594
7. Levy, G. G., Nichols, W. C., Lian, E. C., Foroud, T., McClintick, J. N., Mcgee, B. M., Yang, A. Y., Siemeniak, D. R., Stark, K. R., Grupp, R., Sarode, R., Shurin, B. S., Chandrasekaran, V., Stabler, S. P., Sabio, H., Bouhaissia, E. E., Upshaw, J. D., Jr., Ginsburg, D., and Tsai, H. M. (2001) Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature 413, 488 – 494
8. Kashiwagi, M., Enghild, J. J., Gendron, C., Hughes, C., Caterson, B., Itoh, Y., and Nagase, H. (2004) Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing. J. Biol. Chem. 279, 10109 – 10119
9. Gendron, C., Kashiwagi, M., Lim, N. H., Enghild, J. J., Thøgersen, I. B., Hughes, C., Caterson, B., and Nagase, H. (2007) Proteolytic activities of human ADAMTS-5. Comparative studies with ADAMTS-4. J. Biol. Chem. 282, 18294 – 18306
10. Fushimi, K., Troeberg, L., Nakamura, H., Lim, N. H., and Nagase, H. (2008) Functional differences of the catalytic and non-catalytic domains in human ADAMTS-4 and ADAMTS-5 in aggregcanolytic activity. J. Biol. Chem. 283, 6706 – 6716
11. Zheng, X., Nishio, K., Majerus, E. M., and Sadler, J. E. (2003) Cleavage of von Willebrand factor requires the spacer domain of the metalloprotease ADAMTS13. J. Biol. Chem. 278, 30136 – 30141
12. Gao, W., Anderson, P. J., Majerus, E. M., Tuley, E. A., and Sadler, J. E. (2006) Exosite interactions contribute to tension-induced cleavage of von Willebrand factor by the antithrombotic ADAMTS13 metalloprotease. Proc. Natl. Acad. Sci. U.S.A. 103, 19099 – 19104
13. Zanardelli, S., Crawley, J. T., Chion, C. K., Lam, J. K., Preston, R. I., and Lane, D. A. (2006) ADAMTS13 substrate recognition of von Willebrand factor A2 domain. J. Biol. Chem. 281, 1555 – 1563
14. Wu, J. J., Fujikawa, K., McMullen, B. A., and Chung, D. W. (2006) Carbohydrate structures of active ADAMTS13 complexes. Proc. Natl. Acad. Sci. U.S.A. 103, 18470 – 18474
15. Zanardelli, S., Chion, A. C., Groot, E., Lenting, P. J., McKinnon, T. A., Laffan, M. A., Tseng, M., and Lane, D. A. (2006) ADAMTS13 substrate recognition of von Willebrand factor A2 domain. J. Biol. Chem. 281, 1555 – 1563
16. Morins, E. A. (2005) Deletion of active ADAMTS5 prevents carilage degradation in a murine model of osteoarthritis. Nature 434, 644 – 648
17. de Groot, R., Bardhan, A., Ramroop, N., Lane, D. A., and Crawley, J. T. (2009) Essential role of the disintegrin-like domain in ADAMTS13 function. Proc. Natl. Acad. Sci. U.S.A. 106, 19274 – 19279
18. Feys, H. B., Anderson, P. J., Vanhoorelbeke, K., Majerus, E. M., and Sadler, J. E. (2009) Multi-step binding of ADAMTS-13 to von Willebrand factor. J. Biol. Chem. 284, 28194 – 28200
19. Zanardelli, S., Crawley, J. T., Chion, C. K., Lam, J. K., Preston, R. I., and Lane, D. A. (2006) ADAMTS13 substrate recognition of von Willebrand factor A2 domain. J. Biol. Chem. 281, 1555 – 1563
20. de Groot, R., Bardhan, A., Ramroop, N., Lane, D. A., and Crawley, J. T. (2009) Essential role of the disintegrin-like domain in ADAMTS13 function. Proc. Natl. Acad. Sci. U.S.A. 103, 18470 – 18474
21. Zanardelli, S., Chion, A. C., Groot, E., Lenting, P. J., McKinnon, T. A., Laffan, M. A., Tseng, M., and Lane, D. A. (2006) A novel binding site for ADAMTS13 constitutively exposed on the surface of globular VWF. Blood 111, 2819 – 2828
22. Akiyama, M., Takeda, S., Kokame, K., Takagi, J., and Miyata, T. (2009) Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. Proc. Natl. Acad. Sci. U.S.A. 106, 19274 – 19279
B. M. (2010) An autoantibody epitope comprising residues R660, Y661, and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. Blood 115, 1640–1649
24. Majerus, E. M., Anderson, P. J., and Sadler, J. E. (2005) Binding of ADAMTS13 to von Willebrand factor. J. Biol. Chem. 280, 21773–21778
25. Geiser, M., Cebe, R., Drewello, D., and Schmitz, R. (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. BioTechniques 31, 88–90, 92
26. Kokame, K., Nobe, Y., Kokubo, Y., Okayama, A., and Miyata, T. (2005) FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br. J. Haematol. 129, 93–100
27. Miller, J. A., Liu, R. Q., Davis, G. L., Pratta, M. A., Trzaskos, J. M., and Copeland, R. A. (2003) A microplate assay specific for the enzyme aggrecanase. Anal. Biochem. 314, 260–265
28. Hughes, C. E., Caterson, B., Fosang, A. J., Roughley, P. J., and Mort, J. S. (1995) Monoclonal antibodies that specifically recognize neoepitope sequences generated by “aggrecanase” and matrix metalloproteinase cleavage of aggrecan. Application to catabolism in situ and in vitro. Biochem. J. 305, 799–804
29. Zhang, P., Pan, W., Rux, A. H., Sachais, B. S., and Zheng, X. L. (2007) The cooperative activity between the carboxyl-terminal TSP1 repeats and the CUB domains of ADAMTS13 is crucial for recognition of von Willebrand factor under flow. Blood 110, 1887–1894
30. Soejima, K., Matsumoto, M., Kokame, K., Yagi, H., Ishizashi, H., Maeda, H., Nozaki, C., Miyata, T., Fujimura, Y., and Nakagaki, T. (2003) ADAMTS-13 cysteine-rich/spacer domains are functionally essential for von Willebrand factor cleavage. Blood 102, 3232–3237
31. Mosyak, L., Georgiadis, K., Shane, T., Svenson, K., Hebert, T., Mackie, T., Olland, S., Lin, L., Zhong, X., Kriz, R., Reifenberg, E. L., Collins-Racie, L. A., Corcoran, C., Freeman, B., Zollner, R., Marvell, T., Vera, M., Sum, P. E., Lavallie, E. R., Stahl, M., and Somers, W. (2008) Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5. Protein Sci. 17, 16–21
32. Longpré, J. M., McCulloch, D. R., Koo, B. H., Alexander, J. P., Apte, S. S., and Leduc, R. (2009) Characterization of proADAMTS5 processing by proprotein convertases. Int. J. Biochem. Cell Biol. 41, 1116–1126
33. Majerus, E. M., Zheng, X., Tuley, E. A., and Sadler, J. E. (2003) Cleavage of the ADAMTS13 propeptide is not required for protease activity. J. Biol. Chem. 278, 46643–46648
34. Lim, N. H., Kashiwagi, M., Visse, R., Jones, J., Enghild, J. J., Brew, K., and Nagase, H. (2010) Reactive-site mutants of N-TIMP-3 that selectively inhibit ADAMTS-4 and ADAMTS-5. Biological and structural implications. Biochem. J. 431, 113–122
35. Pruss, C. M., Notley, C. R., Hegadorn, C. A., O’Brien, L. A., and Lillicrap, D. (2008) ADAMTS13 cleavage efficiency is altered by mutagenic and, to a lesser extent, polymorphic sequence changes in the A1 and A2 domains of von Willebrand factor. Br. J. Haematol. 143, 552–558
36. Xiang, Y., de Groot, R., Crawley, J. T., and Lane, D. A. (2011) Mechanism of von Willebrand factor scissile bond cleavage by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). Proc. Natl. Acad. Sci. U.S.A. 108, 11602–11607
37. Zhang, Q., Zhou, Y. F., Zhang, C. Z., Zhang, X., Lu, C., and Springer, T. A. (2009) Structural specializations of A2, a force-sensing domain in the ultralarge vascular protein von Willebrand factor. Proc. Natl. Acad. Sci. U.S.A. 106, 9226–9231
38. Nicholson, A. C., Malik, S. B., Logsdon, J. M., Jr., and Van Meir, E. G. (2005) Functional evolution of ADAMTS genes. Evidence from analyses of phylogeny and gene organization. BMC Evol. Biol. 5, 11
39. Colige, A., Ruggiero, F., Vandenberghe, I., Dubail, J., Kesteloot, F., Van Beumden, J., Beschin, A., Brys, L., Lapière, C. M., and Nusgens, B. (2005) Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V. J. Biol. Chem. 280, 34397–34408
40. Luken, B. M., Turenhout, E. A., Kaijen, P. H., Greuter, M. J., Pos, W., van Mourik, J. A., Fijnheer, R., and Voorberg, J. (2006) Amino acid regions 572–579 and 657–666 of the spacer domain of ADAMTS13 provide a common antigenic core required for binding of antibodies in patients with acquired TTP. Thromb. Haemost. 96, 295–301
41. Wu, J. J., Fujikawa, K., Lian, E. C., McMullen, B. A., Kulman, J. D., and Chung, D. W. (2006) A rapid enzyme-linked assay for ADAMTS-13. J. Thromb. Haemost. 4, 129–136