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## Cleavage of the ADAMTS13 Propeptide Is Not Required for Protease Activity<sup>\*, □</sup>

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### Abstract

ADAMTS13 belongs to the “a disintegrin and metalloprotease with thrombospondin repeats” family, and cleaves von Willebrand factor multimers into smaller forms. For several related proteases, normal folding and enzymatic latency depend on an NH<sub>2</sub>-terminal propeptide that is removed by proteolytic processing during biosynthesis. However, the ADAMTS13 propeptide is unusually short and poorly conserved, suggesting it may not perform these functions. ADAMTS13 was secreted from transfected HeLa cells with a half-time of 7 h and the rate-limiting step was exported from the endoplasmic reticulum. Deletion of the propeptide did not impair the secretion of active ADAMTS13, indicating that the propeptide is dispensable for folding. Furin was shown to be sufficient for ADAMTS13 propeptide processing in two ways. First, mutation of the furin consensus recognition site prevented propeptide cleavage in HeLa cells and resulted in secretion of pro-ADAMTS13. Second, furin-deficient LoVo cells secreted ADAMTS13 with the propeptide intact, and cotransfection with furin restored propeptide cleavage. In both cell lines, secreted pro-ADAMTS13 had normal proteolytic activity toward von Willebrand factor. In cells coexpressing both ADAMTS13 and von Willebrand factor, pro-ADAMTS13 cleaved pro-von Willebrand factor intracellularly. Therefore, the ADAMTS13 propeptide is not required for folding or secretion, and does not perform the common function of maintaining enzyme latency.

ADAMTS13,<sup>1</sup> a member of the a disintegrin and metalloprotease with thrombospondin repeats family (1), cleaves von Willebrand factor (VWF) subunits between Tyr<sup>1605</sup> and Met<sup>1606</sup> to generate two fragments of 140 and 176 kDa (2, 3). VWF is secreted from endothelial cells and platelets as “unusually large” multimers (4) and the inability to cleave

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□ The on-line version of this article (available at <http://www.jbc.org>) contains sequences.

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<sup>1</sup>The abbreviations used are: ADAMTS, a disintegrin and metalloprotease with thrombospondin repeats; PNGase F, peptide: N-glycosidase F; VWF, von Willebrand factor; MMP, matrix metalloprotease; endo H, endoglycosidase H.

unusually large multimers to smaller sizes results in thrombotic thrombocytopenic purpura, a frequently fatal disorder characterized by disseminated platelet-rich microvascular thrombosis (5, 6). Thrombotic thrombocytopenic purpura can be caused either by congenital deficiency of ADAMTS13 or by the development of inactivating antibodies to it (7, 8).

ADAMTS13 shares similarities in domain structure with other ADAMTS proteases but also has significant differences that make it the most divergent member of the group. The ADAMTS family belongs to the metzincin superfamily of zinc metalloproteases (9), and is composed of 19 proteins that have common structural domains including a hydrophobic signal sequence, a propeptide, a metalloprotease domain, a thrombospondin-1 repeat, a disintegrin-like region, a cysteine-rich domain, and a spacer domain (1, 10–13). Many ADAMTS proteases have additional thrombospondin-1 repeats after their spacer domain, and ADAMTS13 has 7 of them. But in contrast to any other family member, the carboxyl terminus of ADAMTS13 concludes with two CUB domains, which were first identified in the complement proteins C1r and C1s (14). Members of the closely related ADAM family of membrane-associated proteases also have similar propeptide, metalloprotease, and disintegrin domains, but lack thrombospondin-1 repeats and have different characteristic domains appended to the carboxyl terminus (15, 16). The more distantly related matrix metalloproteases (MMPs), also have similar propeptide and metalloprotease domains, although their COOH-terminal motifs are not conserved with ADAMTS proteases (17).

Compared with all other ADAMTS proteases and most ADAMs and MMPs, the ADAMTS13 propeptide is exceptionally short, containing  $\approx 41$  amino acids residues instead of a more typical  $\approx 200$  residues. In other metalloproteases, propeptides may assist protein folding as endogenous chaperones (18–20) or inhibit proteolytic activity by a “cysteine-switch” mechanism in which a conserved Cys residue coordinates the active site  $Zn^{2+}$  ion (18, 21, 22). Such metalloprotease zymogens may be activated by cleavage after a proprotein convertase site with the sequence  $RX(K/R)R$ , liberating the propeptide and exposing the protease active site. As in other ADAMTS proteases, the ADAMTS13 propeptide does terminate in a typical proprotein convertase site,  $RQRR$ , but unlike most other family members ADAMTS13 does not have a potential cysteine-switch motif (11, 12, 23, 24). To determine whether the unique structural features of the ADAMTS13 propeptide reflect distinct functional properties, the role of the propeptide in biosynthesis and enzyme latency was investigated by mutagenesis.

## EXPERIMENTAL PROCEDURES

### Materials—

Anti-propeptide antibody was made in rabbits against the predicted ADAMTS13 propeptide amino acid sequence SPGAPLKGRPPSPGFQRQR (amino acid residues 51–69) with a Cys residue added to the  $NH_2$  terminus (Alpha Diagnostic Int., San Antonio, TX). Rabbit IgG was purified from serum by chromatography on Protein A-Sepharose (Pharmacia Corp.). The IgG fraction was affinity purified on a column of the immunizing peptide linked to Sulfo-Link Coupling Gel (Pierce). LoVo and HeLa cells were from the American Type Culture Collection (Manassas, VA).

### Plasmid Constructs—

A full-length cDNA encoding ADAMTS13 was cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to generate plasmid pADAMTS13 as previously described (11, 25). Mutations were introduced using a QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. The predicted proprotein convertase cleavage site RQRR<sup>74</sup> was changed to KQDR<sup>74</sup> with primer 5-CAGAGGCAGAGGCAGAAGCAGGACCGGGCTGCAGGCGGCATC-3 and its complement, generating plasmid pADAMTS13-R71K/R-73D. The propeptide (amino acid residues Gln<sup>34</sup>-Arg<sup>74</sup>) was deleted with primer 5-TGCTGGGGACCCTCCCATTTCGCTGCAGGCGGCATCCTACAC-3' and its complement, generating plasmid pADAMTS13-delPro. Sequences were verified using dideoxy sequencing (Big Dye V3.0, U. S. Biochemical Corp., Cleveland, OH). Plasmids encoding COOH-terminal truncations of ADAMTS13 were prepared as described previously (25). These included constructs in which the V5 epitope and poly(His) tags were inserted after ADAMTS13 amino acid residues Gln<sup>289</sup>, Gly<sup>385</sup>, Glu<sup>429</sup>, Cys<sup>555</sup>, or Ala<sup>685</sup> were cloned in pcDNA3.1D/V5-His-TOPO (Invitrogen); or after Tyr<sup>745</sup>, Arg<sup>807</sup>, Ala<sup>894</sup>, Pro<sup>952</sup>, Arg<sup>1015</sup>, Arg<sup>1075</sup>, and Ala<sup>1191</sup> were cloned in pcDNA3.1/V5-His-TOPO (Invitrogen). Full-length furin cDNA was cloned from a human umbilical vein endothelial cell gt11 cDNA library (26). Oligonucleotide primers based on the furin cDNA sequence (27) were used to amplify a 3' 1.4-kb fragment by PCR, and this product was used to screen the library by DNA hybridization. A full-length cDNA insert was identified and cloned into plasmid pCMV, yielding plasmid pFurin. Plasmid pSVHVWF1.1 encodes full-length human VWF (28).

### Transient Transfection of HeLa and LoVo Cells—

Cells were split into T25 flasks the day before transfection and replated at ~50% confluence. They were transfected using 8  $\mu$ l of LipofectAMINE and 12  $\mu$ l pf Plus reagent (Invitrogen) and 5  $\mu$ g of DNA in 500  $\mu$ l of Opti-MEM (Invitrogen) according to the manufacturer's directions. Media and cell lysates were collected at 48 h post-transfection. To prepare lysates, cells were washed and scraped in phosphate-buffered saline and lysed on ice in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate), and insoluble material was removed by centrifugation at 14,000  $\times$  *g* for 10 min. Recombinant protein samples were concentrated by methanol-chloroform precipitation (29) or by immunoprecipitation with anti-V5 antibody (LoVo cells). Equivalent fractions of the media and cell lysate were subjected to SDS-PAGE and transferred by electroblotting onto Immobilon P (Millipore). ADAMTS13 proteins were detected with monoclonal peroxidase-conjugated anti-V5 antibody (Invitrogen) diluted 1:5000 or affinity purified anti-propeptide antibody diluted 1:100 followed by peroxidase-conjugated swine anti-rabbit IgG (DAKO Corp., Carpinteria, CA), and the chemiluminescent ECL detection system (Amersham Biosciences). VWF was detected similarly with rabbit polyclonal horseradish peroxidase-labeled anti-human VWF antibody P226 (Dako Corp.).

### Pulse-Chase Analysis of ADAMTS13 Synthesis—

HeLa cells were transiently transfected as described above. At 48 h, the cells were washed with phosphate-buffered saline and incubated for 60 min in Dulbecco's modified Eagle's medium (Invitrogen) without methionine or cysteine supplemented with 10% dialyzed fetal calf serum; 300  $\mu\text{Ci/ml}$  Tran<sup>35</sup>S-label™ (ICN) was added and the cells were incubated for another 60 min. The medium was then replaced with Opti-MEM I (Invitrogen) and the cells were incubated at 37 °C for the indicated times of chase. Conditioned media and cell lysates were prepared and equivalent fractions were precleared by incubation with 30  $\mu\text{l}$  of Protein A-Sepharose (RepliGen, Cambridge, MA) in a total volume of 0.5 to 1.0 ml for 1 h at 4 °C, and immunoprecipitated with 1  $\mu\text{l}$  of anti-V5 antibody and 30  $\mu\text{l}$  of Protein A-Sepharose overnight at 4 °C. The beads were washed sequentially with RIPA buffer and 10 mM Tris-HCl, pH 7.5. Bound proteins were eluted by boiling in SDS-PAGE sample loading buffer (15 mM Tris-HCl, pH 6.8, 2.5% glycerol, 0.5% SDS, 178 mM  $\beta$ -mercaptoethanol, and 0.25% bromophenol blue). The eluate was diluted with 50 mM sodium citrate, pH 5.5 (for endoglycosidase H), or 50 mM sodium phosphate, pH 7.5 (for peptide *N*-glycosidase F), such that the SDS concentration was <0.2% and equal fractions were incubated without or with 1000 units of *Streptomyces plicatus* endoglycosidase H (endo H) or 1000 units of peptide: *N*-glycosidase F (New England Biolabs) at 37 °C for 1–2 h. The products were separated by 5% SDS-PAGE. The gel was fixed in 25% 2-propanol, 10% acetic acid, incubated in Amplify™ (Amersham Biosciences), dried, and exposed to Kodak X-AR film at –70 °C.

### Assay of ADAMTS13 Activity—

Activity was assayed based on modifications of the methods of Tsai (3) and Furlan *et al.* (2). Samples were diluted into buffer such that the final concentration was 1 M urea, 5 mM Tris-HCl, pH 8.0, with or without 5 mM EDTA. VWF was added to a final concentration of 1.5  $\mu\text{g/ml}$ , and incubated at room temperature for 1–2 h. Products were analyzed by SDS-PAGE, blotting onto Immobilon P, incubation with horseradish peroxidase-conjugated polyclonal rabbit anti-VWF (number P226, Dako), and chemiluminescent detection with the ECL detection system (Amersham Biosciences). Films were scanned and densitometry was performed using NIH Image 1.62 (developed at the National Institutes of Health and available on the Internet).<sup>2</sup> Protease activity was measured by comparing the intensity of the homodimeric 350-kDa product band for reactions performed in the absence and presence of EDTA.

## RESULTS

### Time Course of ADAMTS13 Glycosylation and Secretion—

ADAMTS13 biosynthesis was examined to provide a framework for understanding the effects of propeptide mutations. In previous studies, the stable expression of ADAMTS13 in several mammalian cell lines was associated with low levels of protease secretion compared with transiently transfected COS-7 or COS-1 cells (25). Therefore, transient transfections were performed with several cell lines including HepG2, RFL6, COS-1, and HeLa. The highest expression levels were obtained with HeLa cells (data not shown), which then were used to assess the time course of ADAMTS13 glycosylation and secretion (Fig. 1).

Pulse-labeled ADAMTS13 appeared in the medium within 3 h of chase with a half-time for secretion of  $\approx 7$  h. No radiolabeled ADAMTS13 could be detected within the cell after 35 h (data not shown), and secreted ADAMTS13 appeared to be stable in the culture medium.

ADAMTS13 purified from plasma is a glycoprotein (23) and recombinant ADAMTS13 contains peptide: *N*-glycosidase F (PNGase F)-sensitive *N*-linked oligosaccharides (30). The protein has 10 potential *N*-glycosylation sites, and to evaluate their status lysates from HeLa cells expressing full-length ADAMTS13 and COOH-terminal truncated variants were digested with endo H. The results indicate that the following sites are utilized: 2 in the metalloprotease domain, 1 in the Cys-rich domain, 2 or 3 in the spacer domain, and 1 in the second thrombospondin-1 repeat. A potential site in the fourth thrombospondin-1 repeat and one in each of the two CUB domains could not be assessed by this approach (data not shown). In addition, sialidase treatment increased the electrophoretic mobility of secreted ADAMTS13 (Fig. 2), but not if first digested with PNGase F (data not shown), suggesting that sialic acid is attached mainly to *N*-linked oligosaccharides. The *N*-linked oligosaccharides on secreted ADAMTS13 were resistant to endo H (Fig. 1, *lanes 8, 12, and 17*) suggesting that all or nearly all have a complex-type structure. In contrast, ADAMTS13 in cell lysates was sensitive to endo H digestion throughout the 24 h of chase (*lanes 2, 6, 10, and 14*), indicating that intracellular ADAMTS13 is predominately located in compartments of the secretory pathway prior to the cis-Golgi (31). These results suggest that the rate-limiting step for ADAMTS13 secretion is protein folding within the endoplasmic reticulum.

#### **Furin Consensus Site Is Required for ADAMTS13 Propeptide Cleavage—**

Many metalloproteases are synthesized with a propeptide that may assist in protein folding (18–20) or that may be cleaved to activate the zymogen form of the protease (18, 21, 22). The NH<sub>2</sub>-terminal residue of ADAMTS13 purified from plasma is Ala<sup>75</sup> (12, 23, 24), suggesting that the potential proprotein convertase site after RQRR<sup>74</sup> is cleaved during biosynthesis, but the propeptide of ADAMTS13 is remarkably short and lacks an apparent cysteine-switch motif that might confer latency on pro-ADAMTS13.

To determine whether propeptide cleavage is required for ADAMTS13 proteolytic activity, the potential cleavage site was mutated. Based on previous studies of furin specificity (32), the sequence RQRR<sup>74</sup> was changed to KQDR<sup>74</sup>. Upon expression in HeLa cells intracellular wild-type ADAMTS13 retained the propeptide, whereas secreted ADAMTS13 did not (Fig. 3, *lanes 5 and 6*), which is consistent with cleavage of the propeptide by furin. In contrast, secreted pro-ADAMTS13-R71K/R73D was detected in the media (Fig. 3, *lane 4*), demonstrating that the proprotein convertase consensus site is needed for propeptide cleavage and that removal of the propeptide is not necessary for secretion. As shown by reactivity with the anti-V5 antibody, ADAMTS13-R71K/R73D appeared to be secreted less efficiently than wild-type ADAMTS13 (Fig. 3, *lanes 8 and 9*), suggesting that the R71K/R73D mutations may delay exit from the endoplasmic reticulum in addition to preventing propeptide cleavage.

### **Furin Cleaves the ADAMTS13 Propeptide—**

To obtain additional evidence that furin could be the responsible proprotein convertase, ADAMTS13 was expressed in the LoVo human colon adenocarcinoma cell line, which lacks furin activity (33). ADAMTS13 was secreted with its propeptide intact, confirming that propeptide cleavage is not required for secretion (Fig. 4). When ADAMTS13 and furin were co-expressed the propeptide was no longer detected in secreted ADAMTS13, indicating that propeptide cleavage was restored. As observed for ADAMTS13-R71K/R73D in HeLa cells (Fig. 3), pro-ADAMTS13 appeared to be secreted slightly less efficiently than mature ADAMTS13 made in LoVo cells cotransfected with furin (Fig. 4, *lanes 10 and 11*). Intracellular pro-ADAMTS13 in LoVo cells remained sensitive to endoglycosidase H (data not shown), indicating that decreased secretion is not caused by retention in the trans-Golgi network.

### **Secreted Pro-ADAMTS13 Is Proteolytically Active—**

The unusual structural features of the ADAMTS13 propeptide suggest it may not maintain enzyme latency. Therefore, pro-ADAMTS13 variants made in HeLa or LoVo cells were assayed for the ability to cleave VWF (Fig. 5). The VWF substrate consists of multimers that may exceed 20,000 kDa, which do not consistently enter polyacrylamide gels or transfer to membranes, but cleavage by ADAMTS13 results in the appearance of an easily monitored 350-kDa homodimeric fragment. LoVo cells transfected with ADAMTS13 secrete pro-ADAMTS13, and LoVo cells transfected with both ADAMTS13 and furin secrete mature ADAMTS13 lacking the propeptide (Fig. 4). In either case, the secreted pro-ADAMTS13 and ADAMTS13 cleaved VWF with equal efficiency and, as expected, activity was abolished by chelation of divalent metal ions with EDTA. No activity was detected in the medium of cells transfected with vector alone. To exclude the possibility that pro-ADAMTS13 was cleaved and activated during the assay, a sample was analyzed by SDS-PAGE and immunoblotting with anti-V5 antibody and shown to remain intact after incubation (data not shown). Similar results were obtained for wild-type ADAMTS13 (lacking propeptide) and ADAMTS13-R71K/R73D (with propeptide) expressed in HeLa cells; both proteins cleaved VWF (Fig. 5). Therefore, propeptide cleavage is not necessary for ADAMTS13 activity against VWF under these assay conditions.

### **Propeptide Is Not Required for ADAMTS13 Intracellular Folding—**

The ADAMTS13 propeptide does not maintain enzyme latency, but might promote folding in the endoplasmic reticulum and enable secretion. To test this hypothesis, the nucleotides that encode the propeptide, amino acids 34–74, were deleted from the ADAMTS13 cDNA. In the expressed mutant protein, amino acid residues Met<sup>1</sup>-Phe<sup>33</sup> comprising the signal peptide were juxtaposed to amino acid residue Ala<sup>75</sup>, which is the NH<sub>2</sub> terminus of purified plasma ADAMTS13 (23, 24). Both wild-type ADAMTS13 and ADAMTS13-delPro were secreted efficiently by HeLa cells (Fig. 6A) and were equally active in cleaving VWF (Fig. 6B); therefore, the propeptide is not necessary for folding and secretion of active ADAMTS13.

### Intracellular Pro-ADAMTS13 and ADAMTS13 Are Proteolytically Active—

Proteases that require cleavage by furin for activation would become proteolytically competent only upon encountering furin in the trans-Golgi, whereas ADAMTS13 might be active from the time folding was completed in the endoplasmic reticulum. If so, then coexpression with ADAMTS13 could result in the intracellular proteolysis of a substrate protein such as VWF. This prediction was confirmed by transfection of HeLa cells (Fig. 7). Intracellular pro-VWF was cleaved to yield the expected 176-kDa COOH-terminal fragment by wild-type ADAMTS13, by ADAMTS13-R71K/R73D with a mutated furin cleavage site, and by ADAMTS13-delPro lacking the propeptide. The oligosaccharides of intracellular pro-VWF are endo H-sensitive, indicating that it is located within the endoplasmic reticulum (34). In previous studies, secreted ADAMTS13-del6 truncated after the metalloprotease domain was inactive and ADAMTS13-del2 truncated after the spacer domain was active toward plasma VWF multimers. The intracellular forms of these ADAMTS13 mutant proteins had similar properties in HeLa cells: ADAMTS13-del6 was inactive and ADAMTS13-del2 was active toward pro-VWF (Fig. 7). Cells that expressed active ADAMTS13 and VWF also secreted reduced amounts of VWF that consisted only of small multimers (data not shown).

## DISCUSSION

As is the case for many other proteins, intracellular ADAMTS13 contains predominantly endo H-sensitive *N*-linked oligosaccharides (Fig. 1), suggesting that exit from the endoplasmic reticulum is relatively slow compared with transport through the Golgi and secretion. During biosynthesis, at least 6 of 10 potential *N*-glycosylation sites are modified with complex-type oligosaccharides, which are likely to be sialylated (Fig. 2). Consensus sequences also are present in several thrombospondin-1 repeats for *C*-mannosylation of Trp and *O*-fucosylation of certain Ser/Thr residues (11), but their modification status has not yet been determined. Such extensive glycosylation might contribute to the relatively long 2–3 day half-life of ADAMTS13 in plasma (35) or to substrate recognition. For example, glycosylation of VWF, the ADAMTS13 substrate, was shown previously to determine the plasma half-life of VWF by preventing clearance by the hepatic asialoglycoprotein receptor (36).

Proteases of the metzincin superfamily often have NH<sub>2</sub>-terminal propeptides (9) that may be removed by proteolysis in the course of enzyme activation. For example, the propeptides of all ADAMTSs (1, 37), most ADAMs (15, 16), and many MMPs (17) have potential proprotein convertase sites. Cleavage at these sites has been demonstrated for ADAMTS1, -2, -4, -5, -9, and -12 (10, 22, 38–42), for ADAM9, -10, -12, -15, -17, and -19 (18, 43–47), and for MMP11, -14, -16, and -23 (48–51). The human ADAMTS13 propeptide also has a typical proprotein processing site, RQRR<sup>74</sup>, and propeptide cleavage was prevented either by mutations that are known to impair recognition by furin (Fig. 3) or by expression in furin-deficient LoVo cells (Fig. 4). Cotransfection with furin restored normal processing in LoVo cells, proving that furin can cleave the ADAMTS13 propeptide. LoVo cells contain mRNA for several other proprotein convertases including PACE4, PC6, and PC7 (52–54). Provided the enzymes are functional, inability to process ADAMTS13

suggests that these alternative proteases in LoVo cells cannot substitute for furin. Furin is ubiquitously expressed and seems likely to process the ADAMTS13 propeptide *in vivo*, but this conclusion is provisional because data are not available on the proprotein convertase repertoire of hepatic perisinusoidal cells, where ADAMTS13 reportedly is made (55).

The propeptides of metalloproteases usually maintain the enzymes in an inactive or latent state, and enzyme activation may be linked to proteolytic cleavage of the propeptide. The functional importance of propeptide removal has been shown directly for ADAMTS1 (22), for ADAM12, -17 and -19 (18, 46, 47), and for MMP11, -14, and -16 (48, 49, 51); in each case, blocking propeptide cleavage causes the secretion of a persistently inactive zymogen. In addition, an intramolecular chaperone function has been demonstrated for the propeptides of ADAM12 (18), ADAM17 (19), and MMP14 (20).

The presence of a functional furin cleavage site between the propeptide and metalloprotease domain suggested that ADAMTS13 might also require proteolytic activation. However, pro-ADAMTS13 proved to be active whether obtained by mutation of the furin cleavage site or by expression in furin-deficient cells (Fig. 5). Although unusual, the result is consistent with the lack of a cysteine-switch motif in the propeptide that might inhibit the metalloprotease domain. The human ADAMTS13 propeptide does contain a Cys residue that is not in a cysteine-switch sequence context, and this cysteine is not conserved in ADAMTS13 of other animal species (Fig. 8). In addition, deletion of the propeptide was compatible with the secretion of active ADAMTS13 (Fig. 6), indicating that the propeptide is not required for folding. In contrast to many related proteases that are activated by proprotein convertase cleavage in late Golgi compartments, ADAMTS13 appears to be active from the time folding is completed in the endoplasmic reticulum (Fig. 7).

The unexpected finding that pro-ADAMTS13 has proteolytic activity is consistent with several observations relating to its expression in cultured cells and *in vivo*. Recombinant ADAMTS13 usually is expressed at a relatively modest level in a variety of cell lines. Typical concentrations in conditioned medium have been 0.2  $\mu\text{g/ml}$  when expressed in stably transfected baby hamster kidney, COS-7, or Chinese hamster ovary cells. Levels of only 1–2  $\mu\text{g/ml}$  were achieved in transiently transfected COS-7 cells and in baculovirus-infected Sf9 cells (25). It is possible that the expression of ADAMTS13 in these cells is limited by toxic effects of active intracellular pro-ADAMTS13, which may have some activity toward proteins other than its favored substrate, VWF. Also, because ADAMTS13 can degrade VWF intracellularly (Fig. 7), the assembly of functional VWF *in vivo* would require the two proteins to be expressed in different cells. As expected, ADAMTS13 is expressed by hepatic stellate cells (55), whereas VWF is expressed by endothelial cells (56) and megakaryocytes (57).

ADAMTS13 appears to be an unusual example, perhaps the first, of a metzincin metalloprotease that has no mechanism to enforce latency, so that once folded it is constitutively active. However, this conclusion does not exclude an important biological function for the ADAMTS13 propeptide, whose distinctive structural features are shared among ADAMTS13 from fish (*Fugu rubripes*) and mammals (*Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*) (Fig. 8). These features include the lack of a cysteine-



switch motif, remarkably short length, and the persistence of a proprotein convertase cleavage site. Such conservation indicates that the current structure and properties of ADAMTS13 developed before the divergence of fish and tetrapod lineages at least 360 million years ago (58) and have been preserved largely intact since then. The selectable functions of the propeptide remain unknown, but may reflect interactions of ADAMTS13 that are visible to natural selection *in vivo* but not detected readily in cultured cell systems. For example, the propeptide may influence the efficiency of synthesis, regulate the activity or toxicity of ADAMTS13, or enable it to bind other macromolecules.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## The abbreviations used are:

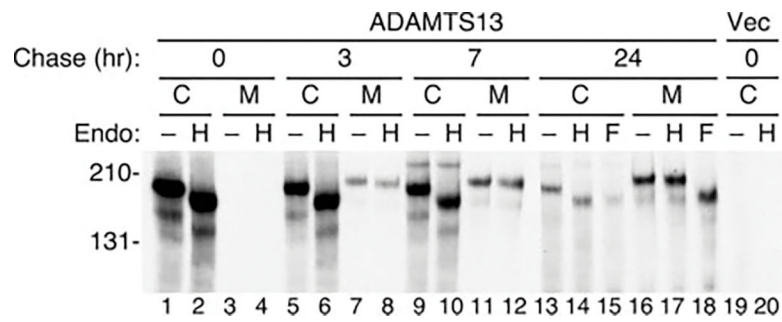
|                 |  |
|-----------------|--|
| <b>ADAMTS</b>   | <u>a</u> <u>dis</u> integrin and <u>met</u> alloprotease with <u>thrombos</u> pondin repeats |
| <b>PNGase F</b> | peptide: <i>N</i> -glycosidase F   |
| <b>VWF</b>      | von Willebrand factor  |
| <b>MMP</b>      | matrix metalloprotease   |
| <b>endo H</b>   | endoglycosidase H  |

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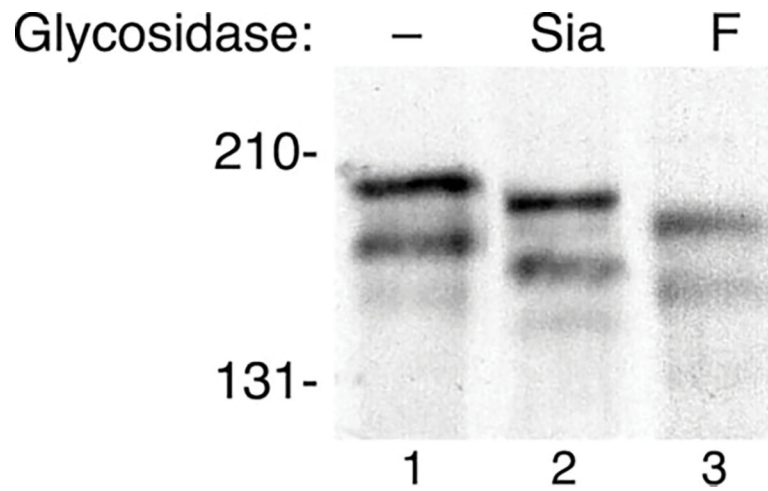
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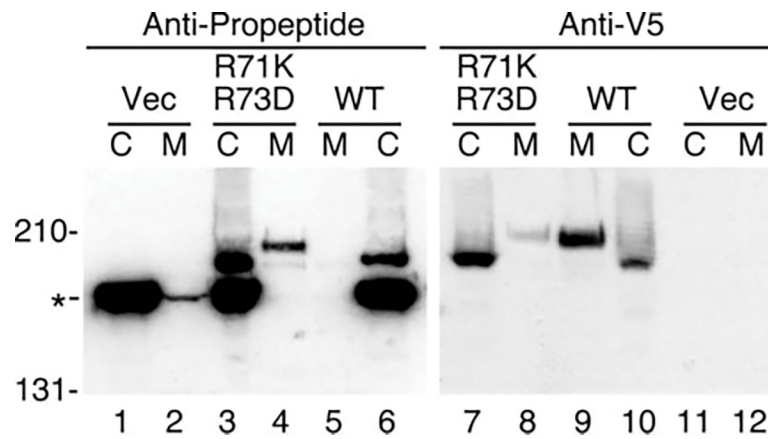
**FIG. 1. Time course of ADAMTS13 secretion and oligosaccharide processing.**

HeLa cells were transfected with pADAMTS13 or pcDNA3.1/V5-His-TOPO vector (*Vec*), pulse-labeled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 1 h, and then incubated for various times of chase. Samples of media (*M*) and cell lysates (*C*) were immunoprecipitated with anti-V5 and treated without (–) or with endoglycosidase H (*H*) or PNGase F (*F*). Products were analyzed by SDS-PAGE and autoradiography. Molecular mass standards in kilodaltons are indicated at the *left*.

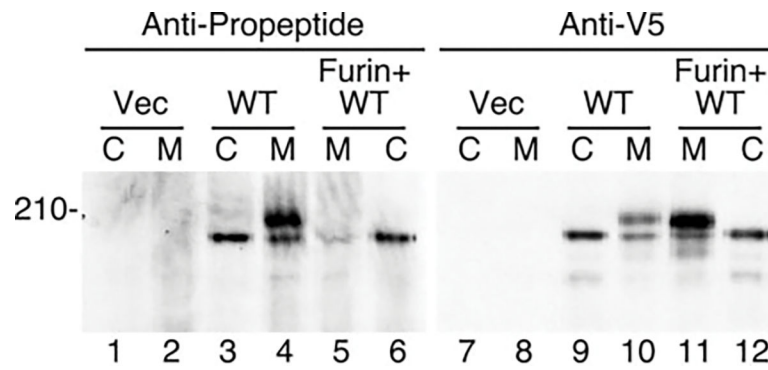


**FIG. 2. Sialidase and PNGase F treatment of secreted ADAMTS13.**

HeLa cells were transfected with pADAMTS13, pulse-labeled with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 1 h, and medium was collected after 6 h of chase. ADAMTS13 was immunoprecipitated with anti-V5 and then treated without (-) or with sialidase A (*Sia*) or PNGase F (*F*). Products were analyzed by SDS-PAGE and autoradiography. The *upper band* in each lane represents full-length ADAMTS13, and faster migrating bands are thought to result from proteolysis. Molecular mass standards in kilodaltons are indicated at the *left*.

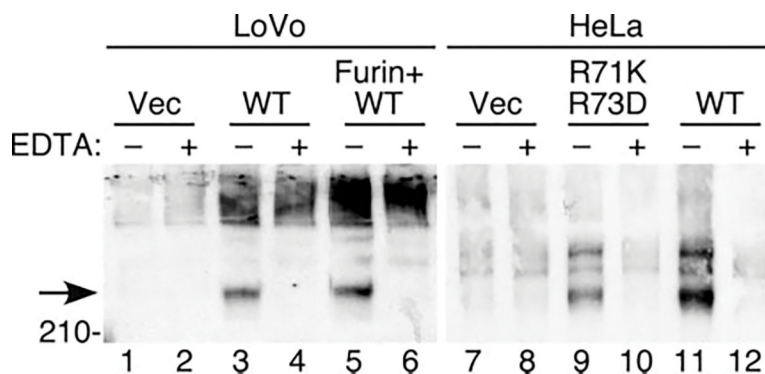


**FIG. 3. Expression of ADAMTS13 with a mutant proprotein convertase cleavage site.** HeLa cells were transfected with vector (*Vec*), pADAMTS13 (*WT*), or pADAMTS13-R71K/R73D with a mutated propeptide cleavage site (*R71K/R73D*). Equivalent fractions of media (*M*) and cell lysates (*C*) were analyzed by SDS-PAGE and Western blotting with anti-propeptide antibody or with anti-V5 antibody. An intense nonspecific band marked with an *asterisk* (\*) was detected with the anti-propeptide antibody in cell lysates. Molecular mass standards are indicated at the *left*.



**FIG. 4. Expression of ADAMTS13 in LoVo cells.**

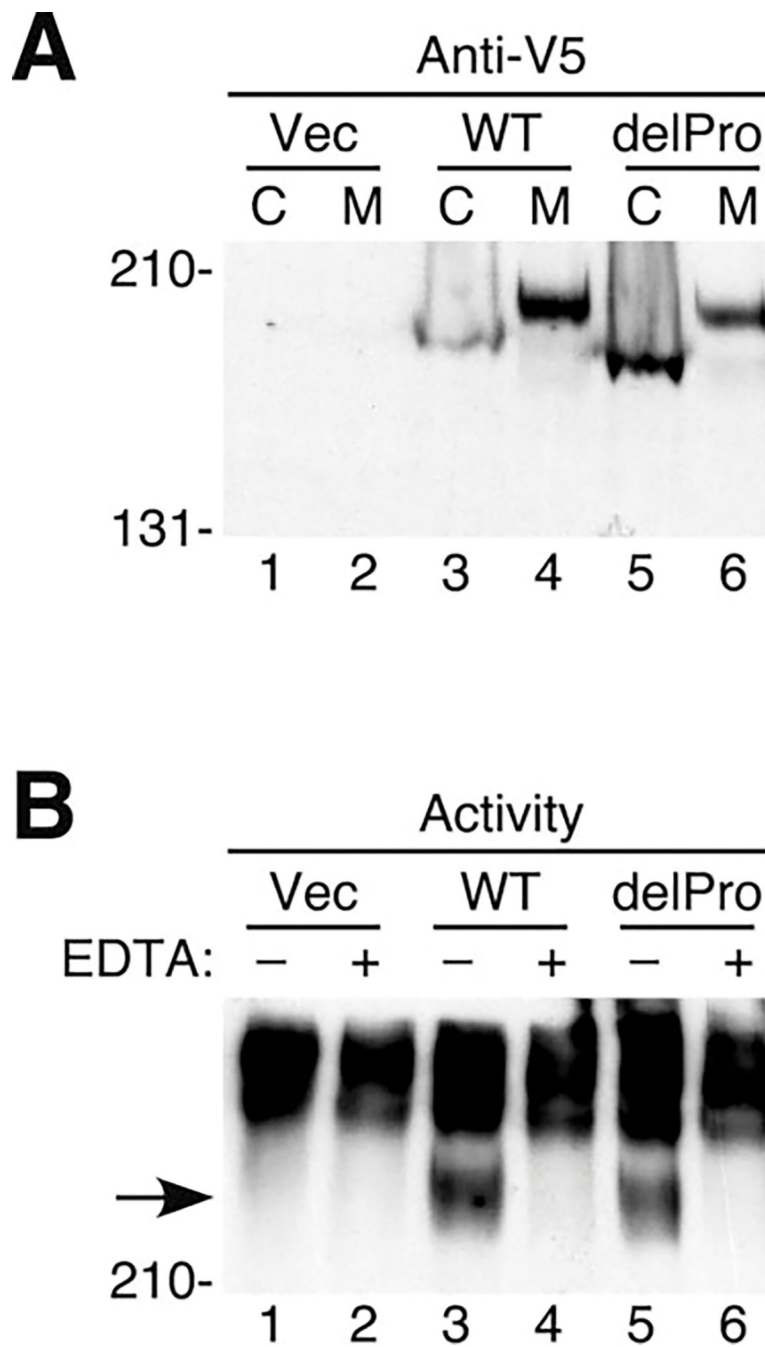
LoVo cells were transfected with vector (*Vec*), pADAMTS13 alone (*WT*), or both pADAMTS13 and pFurin (*Furin*). Media (*M*) and cell lysates (*C*) were analyzed by SDS-PAGE and Western blotting with anti-propeptide antibody (*left*), then stripped and reprobred with anti-V5 antibody (*right*). Molecular mass standards in kilodaltons are indicated at the *left*.



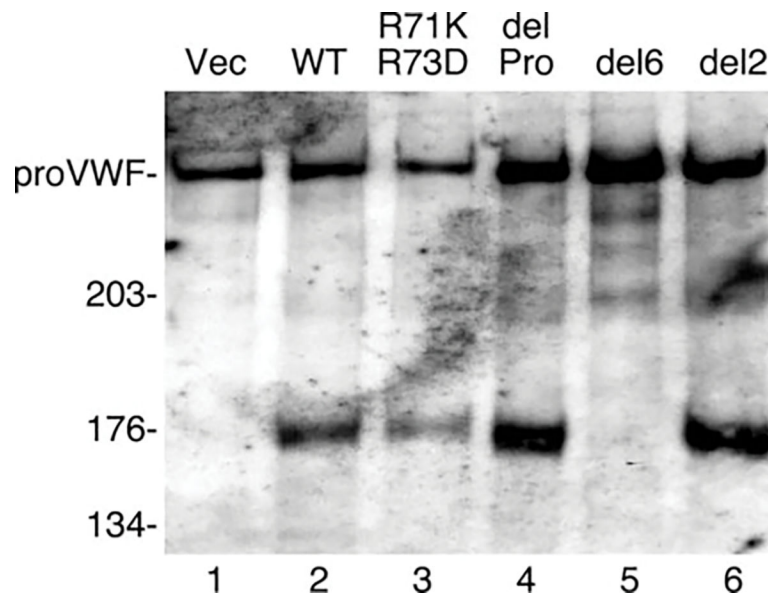
**FIG. 5. Proteolytic activity of pro-ADAMTS13.**

LoVo cells were transfected with vector (*Vec*), pADAMTS13 alone (*WT*), or both pADAMTS13 and pFurin (*Furin*) as in the legend to Fig. 4. HeLa cells were transfected with vector, pADAMTS13, or pADAMTS13-R71K/R73D with a mutated propeptide cleavage site (*R71K/R73D*) as described in the legend to Fig. 3. Samples of media were incubated with VWF for 2 h as described under “Experimental Procedures” in the absence (–) or presence (+) of EDTA. Products were analyzed by SDS-PAGE under nonreducing conditions and Western blotting with anti-human VWF antibody. The position of a molecular mass standard in kilodaltons and the homodimeric 350-kDa cleavage product of VWF (*arrow*) are indicated at the *left*.





**FIG. 6. Secretion and proteolytic activity of ADAMTS13 expressed without its propeptide.** HeLa cells were transfected with vector (*Vec*), pADAMTS13 (*WT*), or pADAMTS13-delPro encoding ADAMTS13 lacking its propeptide (*delPro*). *A*, media and cell lysates were analyzed by SDS-PAGE and Western blotting with anti-V5 antibody. *B*, media were assayed for ADAMTS13 proteolytic activity against VWF in the absence (-) and presence (+) of EDTA. The position of a molecular mass standard in kilodaltons and the homodimeric 350-kDa cleavage product of VWF (*arrow*) are indicated at the *left*.



**FIG. 7. Pro-ADAMTS13 cleaves pro-VWF intracellularly in HeLa cells.**

HeLa cells were cotransfected to express human VWF and a vector control (*Vec*), wild-type pADAMTS13 (*WT*), pADAMTS13-R71K/R73D with a mutated propeptide cleavage site (*R71K/R73D*), pADAMTS13-delPro encoding ADAMTS13 lacking its propeptide (*delPro*), pdel6 encoding ADAMTS13 truncated after the metalloprotease domain (*del6*), or pdel2 encoding ADAMTS13 truncated after the spacer domain (*del2*). VWF was immunoprecipitated from cell lysates and analyzed by SDS-PAGE under reducing conditions and Western blotting with anti-VWF antibody. The positions of molecular mass standards in kilodaltons,  $\approx 360$ -kDa pro-VWF and the 176-kDa COOH-terminal fragment of VWF, are indicated at the *left*.

