Title: N-Glycans of ADAMTS13 Modulate its Secretion and von Willebrand Factor Cleaving Activity

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ABSTRACT

Severe deficiency of ADAMTS13, a plasma metalloprotease, leads to thrombotic thrombocytopenic purpura. ADAMTS13 contains 10 putative N-glycosylation sites in or near its metalloprotease sequence, spacer region, thrombospondin type 1 repeat #4 (TSR #4), and CUB domains. Tunicamycin treatment markedly decreased the secretion of ADAMTS13 into the culture medium of transfected cells. Nevertheless, the protease was efficiently secreted from N-acetylglucosaminyltransferase I deficient Lec1 Chinese hamster ovary cells, indicating that N-glycosylation in the endoplasmic reticulum, but not the conversion of oligomannose to complex N-glycans in the Golgi complex, is important for secretion. However, ADAMTS13 with oligomannose N-glycans cleaved its substrate, von Willebrand factor (VWF) multimers, less effectively, with a higher Km but similar kcat value. In mutagenesis analysis, decreased secretion and VWF cleaving activity was observed with the N146Q and N828Q mutants, while decreased secretion only was observed with the N552Q mutant of ADAMTS13. Enzymatic removal of N-glycans from ADAMTS13 did not affect its VWF cleaving activity. Thus, N-glycosylation is necessary for efficient secretion of ADAMTS13, while conversion of the N-glycans from oligomannose to complex type in the Golgi complex enhances the proteolytic activity of protease toward VWF multimers. After its secretion, ADAMTS13 does not require N-glycans for its VWF cleaving activity.
INTRODUCTION

ADAMTS13, a member of the ADAMTS (a disintegrin and metalloprotease with thrombospondin type 1 repeat) metalloprotease family, cleaves von Willebrand factor (VWF) when this platelet aggregating protein becomes conformationally unfolded by shear stress in the circulation or in vitro by chaotropic agents such as guanidine chloride or urea 1. This proteolysis decreases the adhesive activity of VWF, thereby preventing unwarranted platelet aggregation in the microvasculature. Severe deficiency of ADAMTS13, due to genetic mutations or autoimmune inhibitory antibodies, leads to thrombotic thrombocytopenic purpura (TTP), a serious disorder with characteristic arteriolar and capillary thrombosis and extremely high risk of death if it is not properly treated with plasma therapy to replenish the protease.

ADAMTS13 protein consists of a series of domains, including a signal peptide, a propeptide, a metalloprotease domain, a disintegrin domain, a thrombospondin type 1 repeat (TSR), a cysteine-rich region, a spacer domain, 7 additional TSR’s (#2 - #8), and 2 CUB (complement component Clr/Cls, Uegf, bone morphogenetic protein 1) domains 1. The mature ADAMTS13 protein consists of 1353 amino acid residues but has a molecular weight of 190 kilo Daltons (kDa) as determined by either gel filtration or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), substantially higher than the expected 145 kDa. This large difference suggests that ADAMTS13 is highly glycosylated. In glycoproteins, the glycans attached to the polypeptide backbone via covalent N- or O-linkage may regulate structure and biological functions. Glycosylation may promote appropriate folding, assembly or targeting of nascent proteins and/or their biological activity 2-4. A recent study demonstrates that the N-glycans of VWF have a modulatory effect on its interaction with ADAMTS13 5. Separately, ADAMTS13 has been shown to be O-fucosylated in at least six TSR repeats 6. O-fucosylation is required for secretion of the protease. In this study, we have investigated the role of N-glycans in the secretion and VWF cleaving activity of ADAMTS13.

MATERIALS AND METHODS

Plasmid Constructs and Site-directed Mutagenesis - pcDNA3.1/V5-His plasmids for expression of human ADAMTS13 or its truncated forms with C-terminal V5 and His6 tags were constructed as described previously 7. Mutations of specific amino acids were introduced into ADAMTS13 by PCR site-directed mutagenesis. The mutant constructs were confirmed by sequencing on both strands across the mutated site.

Cell Cultures and Transfections - The Pro-5 Chinese hamster ovary (CHO) parent cell line and the CHO Pro-5 Lec1.3C mutant, which lacks N-acetylglucosaminyltransferase I (GlcNAc-T-I) activity and is termed Lecl in this paper, were isolated previously 8,9. Both lines were grown on 10-cm plates in alpha-modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% (v/v) fetal bovine serum in 5% (v/v) CO2 at 37°C. HEK 293T cells were maintained in Dulbecco’s modified Eagle Medium (DMEM) as previously described 7. Subconfluent cells were transiently transfected in plates with ADAMTS13 plasmids using Lipofectamine 2000 7. To suppress N-glycosylation, HEK 293T cells were treated with 2 μg/mL tunicamycin (Sigma, St. Louis, MO) 24 hours before transfection was performed and maintained in serum free culture medium containing the same concentration of tunicamycin. The conditioned serum-free media were collected at 72 hours and concentrated 30-fold on a Centricon YM-30 filter. The cells were collected in an equal volume of SDS-PAGE sample buffer and stored at -70°C.

Deglycosylation, SDS-PAGE and Immunoblotting - N-glycans were removed from proteins using recombinant peptide N-glycanase F (PNGase F, Prozyme/Glyco, San Leandro, CA) or endoglycosidase H (Roche Applied Science, Indianapolis, IN). Digestion with PNGase F was performed overnight at 37°C in the presence of 0.05 mmol/L EDTA, 0.1 g% SDS and 50 mmol/L β-mercaptoethanol according the manufacturer’s recommendation. In experiments involving analysis of VWF cleaving activity after the digestion step, the incubation was performed in the absence of EDTA, SDS and β-mercaptoethanol. In some experiments, PNGase F was subsequently removed by chromatography over a Sephadex G-100 column (GE Bio-Sciences, Piscataway, NJ). Digestion of ADAMTS13 by endoglycosidase H was performed overnight.
at 37°C in a 0.1 mol/L phosphate buffer, pH 6.0, containing 2 mmol/L EDTA, 0.1 g/mL SDS and 50 mmol/L β-mercaptoethanol. Recombinant ADAMTS13 proteins were separated in SDS-PAGE and visualized by immunoblotting with monoclonal anti-V5 antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and chemiluminescence as previously described⁷. The molar concentrations of recombinant ADAMTS13 proteins were estimated by comparing their respective optical densities against a reference curve constructed from serial dilutions of an AD5 protein (consisting of residues 1-745 of ADAMTS13, Figure 1), whose concentration had previously been calibrated¹⁰. Percentage of protein secretion was defined as the concentration in the culture medium divided by the concentrations in the culture medium and cell lysate x 100%. To compare the efficiency of ADAMTS13 protein secretion, all the protein secretion percentages were normalized against 100% for the mean of the wild type ADAMTS13.

**Measurement of ADAMTS13 Activity** - Measurement of ADAMTS13 proteolytic activity toward VWF multimers was based on the generation of a dimeric 176-kD fragment resulting from cleavage of VWF at the Y1605-M1606 bond as previously described⁷. Briefly, VWF multimers isolated from normal human plasma and dissolved in 1.5 mol/L guanidine chloride was incubated at a ratio of 1:9 (v/v) with protease samples at 37°C. The reaction was terminated at 60 minutes with 4 volumes of SDS PAGE sample buffer containing 5 mmol/L EDTA. The proteolytic fragments of VWF were analyzed in 5% SDS PAGE under non-reducing conditions, followed by immunoblotting with polyclonal rabbit anti-human VWF, horseradish peroxidase conjugated goat anti-rabbit IgG, and chemiluminescence. The activity level of an ADAMTS13 protease, represented by the optic density of the dimeric 176-kD fragment intrapolated against a reference curve constructed from serial dilutions of normal human plasma (NHP, pooled from 10 normal volunteer donors), was expressed in U/nmol protease, with the amount of activity in one milliliter of NHP defined as one unit (U). For comparison among the recombinant proteases, the ADAMTS13 activity values were depicted as a percentage of the activity level in the wild type ADAMTS13.

To measure the values of Michaelis-Menten constant (Km) and catalytic rate constant (kcat) of a protease, reaction of a protease with varying concentrations of VWF multimer substrate was stopped at designated time points. The level of the dimeric 176-kD VWF fragment in the mixtures was determined by SDS PAGE and immunoblotting as in the ADAMTS13 activity assay. The velocity of fragment increase was fitted to the Michaelis-Menten equation as previously described¹⁰. Since the VWF fragment level was not measured in molar concentration, this analysis yielded equivalent Km and kcat values that are suitable only for intra-assay comparison. Consequently, the kinetic values were depicted as a percentage of the corresponding values obtained with the wild type ADAMTS13 protein. Since the size of VWF multimers affected the proteolytic response, all comparisons were based on measurements using the same lot of VWF and analyzed on the same blots. The kinetic analysis was also performed as previously described against FRETS-VWF73 (Peptides International, Louisville, KY) as the substrate at varying concentrations¹⁰. The reaction velocity was determined from the increase of fluorescent intensity measured at intervals of 5 minutes for up to 60 minutes.

**Statistics** - All results are expressed as mean ± standard deviation (SD) unless otherwise indicated. The difference among experimental groups was analyzed by ANOVA and Dunnett post test against the indicated controls. Statistical and curve fitting analyses were performed using Prism (GraphPad Softwares Inc., La Jolla, CA). Difference between two proteases in Km or kcat was determined by using extra sum-of-squares F-test.

**RESULTS**

**ADAMTS13 contains complex N-glycans**

Analysis using artificial neural networks (http://www.cbs.dtu.dk/services/NetNGlyc/) that examine the sequence context of the N-X-S/T sequons revealed that full-length ADAMTS13 contains eight N-X-S/T sequons with N-glycosylation potential above the threshold of 0.5. Two of these sequons (N142 and N146) are in the metalloprotease domain, five (N552, N579, N614, N667 and N707) are in or near the spacer domain, and one (N828) is in the TSR #4 domain (Figure 1A). Two additional candidate residues (N1235 and N1354) present in the CUB domains have glycosylation potentials slightly less than the threshold of 0.5.
To determine whether ADAMTS13 contains N-glycans in these regions, we treated recombinant wild type ADAMTS13 (WT, residues 1-1427) and 4 truncated forms, AD2 (residues 1-448), AD5 (residues 1-745), AD8 (residues 556-745) and AD13 (residues 1016-1427) with PNGase F. SDS-PAGE analysis revealed that all forms in both cell lysates and medium concentrates were reduced in molecular weight by PNGase F (Figure 1B). Therefore, all forms of ADAMTS13 carried N-glycans of the complex or oligomannose type. To distinguish between these types of N-glycans, the ADAMTS13 proteins were treated with endoglycosidase H, which cleaves only oligomannose N-glycans. AD5 and WT ADAMTS13 in cell lysate were digested by endoglycosidase H to the same size generated by PNGase F digestion, indicating that intracellular AD5 and WT ADAMTS13 contained predominantly oligomannose N-glycans and were primarily located in the endoplasmic reticulum or cis Golgi compartment. Both forms of ADAMTS13 were converted to complex N-glycans before secretion as shown by the resistance of the proteins in culture medium to digestion with endoglycosidase H (Figure 1B). The other forms of ADAMTS13 (AD2, AD8 and AD13) were also secreted with complex N-glycans sensitive to removal by PNGase F but not by endoglycosidase H (Figure 1B), as would be expected from the results with AD5 and WT. Taken together, these findings suggest that complex N-glycans are present in and near the metalloprotease, spacer, TSR #4, and CUB domains of the recombinant ADAMTS13. Analysis of 8 N-glycan site mutants (N142Q, N146Q, N552Q, N579Q, N614Q, N667Q, N707Q and N828Q) in HEK 293T secretion by SDS PAGE under reducing conditions showed that each N to Q mutation decreased the molecular weight of ADAMTS13 by approximately 5 kDa (Figure S1), suggesting that each of the N residues was indeed glycosylated in the wild type ADAMTS13.

N-glycosylation is necessary for efficient secretion of ADAMTS13

To determine whether N-glycosylation is essential for the secretion of ADAMTS13, the recombinant glycoproteins were expressed in the presence of tunicamycin, which blocks N-glycosylation by inhibiting the activity of dolichol-P:N-acetylglucosamine phosphotransferase. SDS-PAGE gel analysis shows that in the presence of tunicamycin, WT ADAMTS13, AD5 or AD2 were synthesized at lower levels and had lower molecular weights (Figure 2A). Furthermore, no secreted forms were detected in the medium of tunicamycin-treated cells, indicating that tunicamycin profoundly decreased the secretion of these proteins. The suppressive effect of tunicamycin on the secretion of AD2 suggests that one or both of the N-glycans in the metalloprotease domain may be critical for effective ADAMTS13 secretion.

To determine whether any of the asparagine (N) residues or its glycosylation plays a role in the effective secretion of ADAMTS13, we substituted each of the eight N residues in or near the metalloprotease or spacer domains with glutamine (Q), and compared the secretion of the mutants with that of the wild type ADAMTS13 (WT). This analysis revealed that, compared to the wild type ADAMTS13, three (N146Q, N552Q and N828Q) mutants were secreted somewhat less efficiently (Figure 2B, P<0.01). Nevertheless, individual N residue substitution alone was not sufficient to reproduce the profound decrease of secretion observed with tunicamycin, suggesting that more than one N-glycosylation contributes to the efficient secretion of ADAMTS13.

ADAMTS13 with oligomannose N-glycans are efficiently secreted but are less active

To determine whether conversion of N-glycans from the oligomannose to complex type affects the secretion or biological function of ADAMTS13, we expressed full length ADAMTS13 (WT) and its truncated variants (AD2 and AD5) in N-acetylg glucosaminyltransferase I-deficient cells (Lec1 CHO mutant). Lec1 cells do not convert oligomannose N-glycans to complex N-glycans due to an inactivating mutation in the Mgat1 gene11. The efficiency of secretion of AD2, AD5 and WT ADAMTS13 from Lec1 cells was compared with that from parent CHO cells. As illustrated in Figure 3, there was no apparent difference in the efficiency of secretion from CHO versus Lec1 cells. As expected, the proteins secreted from Lec1 cells were equally sensitive to digestion with both PNGase F and endoglycosidase H and gave forms of the same molecular weight, confirming the presence of solely oligomannose N-glycans on the proteins secreted from Lec1 cells.
The enzymatic activity of AD2, AD5 and WT ADAMTS13 was analyzed using VWF multimers as the substrate. As shown in Figure 4A, WT and AD5 in secretions from Lec1 cells were less effective at cleaving VWF multimers, exhibiting 30%-50% of the wild type activity. This lower activity was consistently observed whether the assay incubation reaction was terminated at 3, 10, or 20 minutes (data not shown) instead of the usual 60 minutes. AD2 expressed in Lec1 cells also exhibited a trend toward lower proteolytic activity. Nevertheless, because of the low activity of AD2, this difference did not reach statistical significance.

The lower VWF cleaving activity observed with both WT/Lec1 and AD5/Lec1 suggested that the N-glycans in the metalloprotease-spacer-TSR #4 region contribute to the proteolytic activity of ADAMTS13. To further explore this possibility, we analyzed the VWF cleaving activity of ADAMTS13 with substitution at one of the eight N residues in this region (Figure 4B). The VWF multimer cleaving activity, in U/nmol protein for each protease derived from HEK 293T cell secretion, was normalized to 100% for the WT ADAMTS13. The results revealed that the N146Q and N828Q mutants were 30% - 50% as active as wild type ADAMTS13 in cleaving VWF multimers (P<0.05). None of the other mutants showed a significantly diminished VWF cleaving activity.

Michaelis-Menten analysis of the VWF cleaving reaction using a previously described procedure showed that the lower proteolytic activity of the N146Q mutant was primarily due to a higher Km (641% of the wild type, P<0.05) while the kcat was not significantly different (86% of wild type) (Figure 5A). No such difference was observed between WT and N146Q mutant when FRETS-VWF73 was used as the substrate (Figure 5B). Similar results were obtained with the N828Q mutant (data not shown).

Similar kinetic analysis revealed that the Km of the full length ADAMTS13 in Lec1 cell secretion was 525% compared to the protease in CHO cell secretion, while the kcat was not significantly different (83% of wild type). The oligomannose variant of ADAMTS13 from Lec1 cell secretion exhibited no difference from the complex type ADAMTS13 from wild type CHO cell secretion in either Km (116% of the wild type) or kcat (122% of the wild type) when FRETS-VWF73 was used as the substrate.

**Presence of N-glycans is not necessary for the VWF cleaving activity of ADAMTS13**

To determine if the N-glycans are necessary for the VWF cleaving activity of ADAMTS13, we treated WT ADAMTS13 with PNGase F in the absence of EDTA, SDS and β-mercaptoethanol. SDS PAGE analysis of the digested ADAMTS13 showed that this modified incubation led to same removal of the N-glycans as the original incubation (Figure 6A). The VWF cleaving activity analysis shows that N-deglycosylation by PNGase F does not affect the VWF cleaving activity of ADAMTS13 (Figure 6B). In the VWF cleaving assay, the VWF fragments generated from cleavage by PNGase F treated ADAMTS13 or its buffer control were not different in molecular size, indicating that PNGase F in the reaction mixtures did not cause N-deglycosylation of the VWF substrate during the assay (data not shown).

**DISCUSSION**

ADAMTS13 is a highly glycosylated plasma protease, with N- and O-glycans accounting for ~20% of its molecular mass. Recent work by Ricketts et al demonstrated that O-fucosylation of the TSR domains is important for secretion of ADAMTS13. In this study, we find that N-glycosylation is also critical for secretion of the protease, since no ADAMTS13 proteases were detected in culture media of cells treated with tunicamycin. On the other hand, the data from N-glycan site mutants of ADAMTS13 suggest that individually, each N-glycan has minimal or moderate impact on the efficiency of secretion. Thus, the dramatic impact of tunicamycin on ADAMTS13 secretion reflects the abolition of more than one N-glycans. A similar phenomenon was observed with O-fucosylation of the TSP repeats. Both N-glycan addition and O-fucosylation occur co-translationally. Early glycosylation may promote ADAMTS13 secretion by ensuring correct protein folding, disulfide bonding, or other processing steps in the endoplasmic reticulum.

In the expression experiments, the intracellular pool of ADAMTS13 consisted essentially of oligomannose N-glycans in the endoplasmic reticulum or cis Golgi. Thus, the ER folding and/or Golgi translocation and early trafficking are likely to be major determinants of ADAMTS13 secretion. In contrast,
ADAMTS13 proteases were secreted efficiently from N-acetylglucosaminyltransferase I deficient Lec1 cells, suggesting that ADAMTS13 exits from the endoplasmic reticulum in a secretion-competent form, and that further processing of oligomannose N-glycans to the complex type in the Golgi is not necessary for secretion.

Previous studies of hereditary TTP have shown that the most common defect of ADAMTS13 mutations is impaired secretion, with retention of mutant proteases in the intracellular compartments. Although none of the known patient mutations affect the N-glycosylation sites, we speculate that exit block from the endoplasmic reticulum may involve a common defect in the signal for secretion. Thus, further investigation of the factors regulating the exit of ADAMTS13 from the endoplasmic reticulum may have important implications for understanding how ADAMTS13 mutations lead to secretion defects.

The predicted N-glycosylation sites of ADAMTS13 are clustered in the central part of the protease, with 5 of them (N552, N579, N614, N667, and N707) located within a 156-residue region of the spacer–TSR #2 domain. One additional site is located slightly downstream in the TSR #4 domain. Interestingly, previous studies using chemically- or shear-stress-unfolded VWF have shown that while truncation of the protease distal to the spacer domain decreases the VWF cleaving activity by approximately 20% - 60%, the residual protease activity is decreased to <2% (but not nil) when the truncation is upstream of the spacer domain. Thus, the spacer domain and, to a lesser extent, the downstream TSR-CUB domains are involved in the expression of VWF cleaving activity. The clustering of N-glycan sites in the spacer-TSR #2 and TSR #4 regions suggested that the N-glycans might affect the proteolytic activity of ADAMTS13. Our analysis of the proteases secreted by Lec1 cells shows that ADAMTS13 with oligomannose side chains are only 30% - 50% as active as the wild type, confirming that N-glycans are functionally important for expression of VWF cleaving activity. Kinetic analysis suggests that the decrease in proteolytic activity of the oligomannose ADAMTS13 results primarily from higher Km, suggesting that conversion of N-glycans from oligomannose to complex type promotes the binding between the protease and VWF. Separately, in solid phase binding analysis we found that in the presence of EDTA, the Lec1 variant of ADAMTS13 was not different from the wild type protease in binding VWF (data not shown). This discrepancy indicates that ADAMTS13-VWF binding data obtained under non-catalytic conditions may not be applied to interpret functional ADAMTS13-VWF interaction.

Surprisingly, our analysis using the N-glycan site mutants reveals that the N residues located in the spacer-TSR #2 domains are not important for expression of proteolytic activity. Instead, N146 in the metalloprotease domain and N828 in the TSR #4 are important for VWF cleaving activity. Notably, both mutants, as well as N552Q, also exhibited secretion defect. Kinetic analysis shows that the lower VWF cleaving activity of the N146Q or N828Q ADAMTS13 mutant results primarily from a higher Km. Interestingly, the N142 residue located 4 residues upstream from N146 does not affect the secretion or proteolytic activity of ADAMTS13. It should be noted that studies using N-glycan site mutants do not distinguish between the effect resulting from loss of N-glycosylation and the effect directly from amino acid substitution.

Further activity analysis of the N-deglycosylated ADAMTS13 shows that removal of N-glycans by PNGase F did not affect the VWF cleaving activity of ADAMTS13. Thus, glycosylation at the N-residues most likely contributes to the ADAMTS13 activity by promoting correct protein folding or disulfide bonding for optimal binding with its VWF substrate. The N-glycans are not needed for VWF cleaving activity once the protease is correctly folded and secreted. In contrast, removal of N-glycans from VWF makes the substrate susceptible to cleavage by ADAMTS13.

The lower proteolytic activity of the N146Q and N828Q mutants and the oligomannose variant ADAMTS13 in Lec1 cell secretion was not reproduced when FRETS-VWF73 was used as the substrate. In a previous report, we observed a similar phenomenon of differential response to the substrates: compared to full length ADAMTS13, truncated ADAMTS13 variants (i.e. AD5 and AD2) exhibited lower binding affinity (i.e. higher Km) for VWF multimers. This difference in Km between full-length ADAMTS13 and its truncated variants markedly decreased when the proteases were assayed against FRETS-VWF73. Taken together, the data of these two studies suggest that N-glycans and the domains downstream of the catalytic site are important for protease-VWF multimer interaction but are less critical when the substrate is an
abbreviated VWF peptide. We speculate that in order to interact effectively with VWF multimers, ADAMTS13 requires a stringent conformation. N-glycosylation and the presence of downstream domains of ADAMTS13 contribute to correct protein folding that is necessary for ADAMTS13 to assume this active conformation. For interaction with short peptide substrate, correct folding of ADAMTS13 is less important.

Analysis of the truncated variants of ADAMTS13 suggests that the recombinant ADAMTS13 is N-glycosylated at the metalloprotease, spacer, TSR #4 and CUB domains. Interpretation of the truncation variant data may be hampered by uncertainty over the impact of truncation mutation on the efficiency of N-glycosylation. Nevertheless, SDS PAGE analysis demonstrates that the each N glycosylation site mutation in these domains decreases the molecular weight of the full length ADAMTS13 by approximately 5 kDa. Together, these observations suggest that all eight N residues are glycosylated in the recombinant ADAMTS13. The two potential N-glycosylation sites in the CUB domain will be investigated in future studies.

It is well recognized that the efficiency of N-glycosylation and the structures of the glycans may be affected by the type of cells synthesizing the protein. Consequently, the ADAMTS13 in plasma, which is believed to derive primarily from hepatic stellate cells and perhaps other types of cells, may be different from recombinant ADAMTS13 proteins in the fine structure of N-glycans. Nevertheless, we find plasma ADAMTS13 and recombinant ADAMTS13 have the same size by SDS PAGE, and both are similarly decreased in size after N-deglycosylation (data not shown). Since the SDS PAGE analysis has the resolution power to detect molecular weight difference resulting from the loss of a single N glycosylation residue, our data suggests that plasma and recombinant ADAMTS13 most likely have the same number of N-glycosylated residues.

In conclusion, N-glycosylation in the endoplasmic reticulum is a critical step for efficient secretion of ADAMTS13. In contrast, the lower proteolytic activity observed with the oligomannose type ADAMTS13 in Lec1 cell secretion suggests that conversion of oligomannose N-glycans to the complex type promotes the transformation of ADAMTS13 to a proteolytically more active conformation. N-glycosylation may contribute to VWF cleaving activity of ADAMTS13 by promoting correct protein folding or disulfide bonding, creating a conformation of ADAMTS13 optimal for interaction with VWF multimers. Nevertheless, once secreted, continued presence of N-glycans is not essential for ADAMTS13 interaction with VWF. N-glycosylation is less important for cleavage of FRETS-VWF73, a short VWF peptide substrate, perhaps because the interaction between ADAMTS13 and the short substrate requires less stringent conformation of the protease. Future studies are needed to determine the precise profile and structure of N-glycans in the plasma ADAMTS13 and the role of N-glycans in the conformational maturation of ADAMTS13 during its biosynthesis.
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References

FIGURE LEGENDS

Figure 1. Expression of ADAMTS13 and its truncation variants in HEK 293T cells. A. The predicted domain structure of ADAMTS13 (WT) and the range of amino acid sequences encompassed in the 5 truncated constructs (AD2, AD5, AD8 and AD13) used in this study. B. Expressed ADAMTS13 and its truncation variants in either cell lysate (Cell) and/or concentrated culture medium (Medium) were digested with PNGase F (F) or endoglycosidase H (H) and analyzed by SDS-PAGE and immunoblotting with anti-V5. C indicates control proteins not digested with either enzyme. The numbers on the left of the gels indicate the position of molecular size markers in kDa. The cell lysate data is not shown for AD2, AD8, and AD13. The space in AD5 and AD13 indicates repositioned gel lanes.

Figure 2. N-glycosylation affects the secretion of ADAMTS13. A. Tunicamycin suppresses the secretion of ADAMTS13. HEK 293T cells were transfected in the absence (B) or presence (T) of tunicamycin. Cell lysates (C) and concentrated culture media (M) were collected 72 hours after transfection and analyzed at equal volumes by SDS-PAGE and immunoblotting with anti-V5. The numbers to the left of the gels indicate the position of molecular weight markers in kDa. B. The effect of N to Q substitution on the secretion of ADAMTS13. Transfection of wild type ADAMTS13 (WT) cDNA or N-glycosylation site mutants was performed in the absence of tunicamycin. Each % ADAMTS13 secretion was determined from the concentration of ADAMTS13 in the culture medium, divided by the sum of protease concentration in the culture medium and cell lysate. The values were normalized against 100% for the mean of the wild type ADAMTS13 secretion. Each mean and standard deviation values shown on the y-axis were based on at least 5 batches of transfections. *P<0.01 by ANOVA with Dunnett post analysis against the wild type ADAMTS13 (WT).

Figure 3. Lec1 cells secreted ADAMTS13 proteins with oligomannose N-glycans. ADAMTS13 (WT) and its truncated variants, AD5 and AD2, were expressed in Chinese hamster ovary cells (CHO) or the Lec1 mutant cells (Lec1). Cell lysates (C) and serum free culture media (M) were collected for SDS PAGE and immunoblotting for V5 tag 72 hours after transfection. Each secreted protein was also digested with PNGase F (F) or endoglycosidase H (H) before analysis. The numbers to the left of each gel indicate the position of the molecular weight markers in kDa. Smaller molecular weight bands in the cell lysates of WT and AD2 might have resulted from proteolysis of expressed protein.

Figure 4. N-glycans affect the VWF cleaving activity of ADAMTS13. A. The mean VWF cleaving activity (± standard deviation) from 4 measurements is depicted for wild type (WT) ADAMTS13, AD5, and AD2 in CHO or Lec1 cell secretion. The insert is magnification of the AD2 results. * indicate P<0.05 by Student’s t-test. B. VWF cleaving activity of wild type ADAMTS13 and 8 N-residue mutants. ADAMTS13 proteins were from secretion of HEK 293T cells. For each mutant, the VWF cleaving activity level per nanomole protein is expressed as a percentage of wild type (WT) ADAMTS13. *P<0.01 by ANOVA with Dunnett post test against the WT ADAMTS13 (N = 4-6).

Figure 5. N146Q mutant is less effective than wild type ADAMTS13 in cleaving VWF multimers but not in cleaving FRETS-VWF73. A. Kinetic analysis of VWF multimer cleavage by wild type
ADAMTS13 (concentration 0.87 nmol/L) or N146Q mutant (concentration 1.08 nmol/L). B. Kinetic analysis of FRETS-VWF cleavage by wild type ADAMTS13 (0.2 nmol/L) or N146Q mutant (0.18 nmol/L). The reaction velocity (WT:♦; N146Q: ◊), based on the increase of the dimeric 176-kD fragment level on immunoblots against a reference curve constructed from serial dilutions of normal human plasma for A and on the increase of fluorescence intensity for B, was curve-fitted to Michaelis-Menten equation (solid and dashed lines) to obtain the equivalent values of Km and $k_{cat}$. Since neither the reaction velocity nor the unit of VWF multimers was measured in molar concentrations, this analysis yielded kinetic value equivalents that were suitable only for intra-assay comparison. The difference between N146Q and WT ADAMTS13 against VWF multimers was significant (<0.05) for Km but not for $k_{cat}$. No significant difference was detected in either Km or $k_{cat}$ against FRETS-VWF73. Similar results were obtained in a repeated analysis.

**Figure 6.** Deglycosylation of ADAMTS13 by PNGase F does not affect its VWF cleaving activity. A. The molecular weight of the wild type ADAMTS13 in HEK 293T cell secretion decreased from 190 kD in the buffer control (B) to 172 kD after overnight incubation at 37°C with PNGase F in the presence (+) or absence (-) of EDTA, SDS and β-mercaptoethanol (β-ME). B. VWF cleaving activity, expressed in % of NHP, of the ADAMTS13 protein assayed at varying concentrations after incubation with buffer (♦) or PNGase F (◊) in the absence of EDTA/SDS/β-ME. NHP: normal human plasma.
Figure 1

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WT (1-1427)

AD5 (1-745)

AD2 (1-448)

AD8 (556-745)

AD13 (1016-1427)

B

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Figure 5

(A) Reaction velocity vs. VWF, µg/mL.

(B) Reaction velocity vs. FRETS-VWF73, µmol/L.
N-glycans of ADAMTS13 modulate its secretion and von Willebrand factor cleaving activity

Wenhua Zhou and Han-Mou Tsai