O-FUCOSYLATION IS REQUIRED FOR ADAMTS13 SECRETION

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SUMMARY

ADAMTS13 is a plasma metalloproteinase that cleaves von Willebrand Factor to smaller less thrombogenic forms. Deficiency of ADAMTS13 activity in plasma leads to thrombotic thrombocytopenic purpura. ADAMTS13 contains eight thrombospondin type 1 repeats (TSR), seven of which contain a consensus sequence for the direct addition of fucose to the hydroxyl group of serine or threonine. Mass spectral analysis of tryptic peptides derived from human ADAMTS13 indicate that at least six of the TSRs are modified with an O-fucose disaccharide. Analysis of [3H]fucose metabolically incorporated into ADAMTS13 demonstrated that the disaccharide has the structure Glucose-β1,3-Fucose. Mutation of the modified serine to alanine in TSRs 2, 5, 7, and 8 reduced the secretion of ADAMTS13. Mutation of more than one site dramatically reduced secretion regardless of the sites mutated. When the expression of protein O-fucosyltransferase 2 (POFUT2), the enzyme that transfers fucose to serines in TSRs, was reduced using siRNA, the secretion of ADAMTS13 decreased. A similar outcome was observed when ADAMTS13 was expressed in a cell line unable to synthesize the donor for fucose addition, GDP-fucose. While overexpression of POFUT2 did not affect the secretion of wild-type ADAMTS13, it did increase the secretion of the ADAMTS13 TSR1,2 double mutant but not that of ADAMTS13 TSR1-8 mutant. Together these findings indicate that O-fucosylation is functionally significant for secretion of ADAMTS13.

INTRODUCTION

ADAMTS13 is a plasma metalloprotease that cleaves von Willebrand Factor to smaller, less thrombogenic fragments. ADAMTS13 is a member of the ADAMTS family of metalloproteases that are characterized by a conserved domain structure. These include a metalloprotease domain, a disintegrin domain, a thrombospondin type 1 repeat (TSR), cysteine-rich domain, a spacer domain and conclude with a variable number of additional TSRs (1). ADAMTS13 uniquely contains seven additional TSRs and two CUB1 domains at its carboxyl end (2-4) (Figure 1A).

TSRs contain approximately 60 amino acids with conserved tryptophans and cysteines. They were first described in thrombospondin type 1 and are homologous to the properdin repeat found in many components of the complement system. Thrombospondin type 1 is a protein that is thought to play a role in angiogenesis, cell adhesion and motility (5,6). Many interactions of thrombospondin-1 are thought to be mediated through amino acids within the TSRs (7). For example, binding of thrombospondin-1 to the endothelial cell protein, CD36, can be inhibited by the peptide CSVTCG which is found in the TSRs of thrombospondin-1 (8). The three TSRs of thrombospondin-1 have been shown to contain a fucose directly linked to a serine or threonine within a putative consensus sequence of $C^1XX(S/T)C^2G$ (where $C^1$ and $C^2$ are the first and second conserved cysteines in the TSR (9)), which is the putative CD36 binding region. The fucose on the TSRs was further modified with a glucose in β1-3 linkage to form a disaccharide (9,10). Subsequent analysis of O-fucosylation on TSRs from properdin and F-spondin resulted in a revised consensus sequence: $WX_3C^1X_2(S/T)C^2X_2G$ (11).

O-fucosylation of proteins has also been described on epidermal growth factor (EGF)-like repeats of many different proteins, including urinary-type plasminogen activator, coagulation factors VII, IX, and XII, and the developmental protein, Notch (12,13). The O-fucose in the EGF-like repeats of factor IX and Notch can be further modified with an N-acetylgalactosamine, galactose, and sialic acid (13,14). Fucosylation and the further elongation of the saccharide is critical for the activity of Notch (15-19). Fucosylation of urinary-type plasminogen activator is also important for the activation of its receptor (20).

The enzyme responsible for the addition of fucose to serines or threonines of EGF-like repeats was identified as protein O-fucosyltransferase 1 (POFUT1) (21). While other known fucosyltransferases are Golgi
resident proteins, POFUT1 is a soluble protein that contains a KDEL-like sequence and localizes to the endoplasmic reticulum (ER) (22,23). POFUT1 only fucosylates properly folded EGF-like repeats, suggesting it may play some role in quality control (24). Studies with Notch have shown that POFUT1 is required for localization of Notch to the plasma membrane (22). Interestingly, an enzymatically inactive mutant of the Drosophila homologue, OFUT1, was able to restore plasma membrane localization of Notch in cells that lacked OFUT1 (22). Along with the ER localization, this result suggests that POFUT1 may have a chaperone role in addition to its fucosyltransferase activity.

POFUT1 is unable to fucosylate TSR repeats, a process mediated by a second protein fucosyltransferase, POFUT2 (10,25). POFUT2 fucosylates TSRs of thrombospondin-1 but not EGF-like repeats. POFUT2 is also localized to the ER but does not contain a KDEL-like sequence (25,26). Interestingly, POFUT2 also only modifies properly folded TSRs, suggesting it too may play some role in quality control (10).

To better understand the role of POFUT2 and fucosylation in TSRs, we have studied the role of fucosylation in the secretion and activity of ADAMTS13, which contains seven potential fucosylation sites in its eight TSRs (Figure 1B). This work examines the functional significance of O-fucosylation in TSRs.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pcDNA4/TO (Invitrogen) and ADAMTS13 in pcDNA 3.1/V5 His (27) were digested with Ndel and HindIII. The 500 nucleotide fragment from pcDNA4/TO containing the tetracycline operon was ligated into the 5000 nucleotide fragment of ADAMTS13 in pcDNA3.1/V5 His to generate a construct with tetracycline-inducible expression and a V5 epitope tag (ADAMTS13 in TOV5). The resulting construct encodes amino acids METDTLLLWVLLLWVPGSTGDASLVPRSSDPLVQCGGIALDYKDDDDK before the POFUT2 sequence of GQFWPG. POFUT2 E395A/E396A was generated using site-directed mutagenesis as previously described and the primers listed in Table 1. pSVH-D’D3pro-c-Myc, which encodes the signal sequence, and D’D3 domains of von Willebrand Factor, was a gift of Dr. Angie Purvis (28).

**Cell lines**—Parental HEK293 TREx cells (Invitrogen) were grown in Dulbecco’s modified Eagle’s media with 10% tetracycline-free fetal bovine serum (Clontech) and 5 µg/ml blasticidin. Lec13-CHO cell line was generated by Dr. Pamela Stanley (29) and were grown in α-Minimal Essential Media containing 10% fetal bovine serum in the presence or absence of 1 mM L-fucose (Sigma).

[^H]fucose labeling and glycosidase digestion of ADAMTS13—HEK293 TREx cells stably-expressing ADAMTS13 (30) were incubated with 50 µCi/ml L-[6-^3H]fucose (American Radiolabeled Chemicals) in Opti-Mem (Invitrogen) and 1 µg/ml tetracycline overnight. The medium was collected and centrifuged in
the presence of 1 µl/ml Protease Inhibitor Cocktail (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were washed with phosphate-buffered saline and lysed in 1 ml radio-immune precipitation assay buffer (RIPA) buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) on ice for greater than 10 minutes. Insoluble cellular material was pelleted by centrifugation at 14,000 X g for ten minutes. Anti-V5 antibody and Protein G-Sepharose were added to equivalent fractions of the cell lysate and medium for immunoprecipitation. The immunoprecipitates were prepared for Peptide: N-glycanase F digestion following the manufacturer’s directions (New England Biolabs). Briefly, the immunoprecipitates were incubated in denaturing buffer at 100°C for five minutes and then, sodium phosphate pH 7.5, Nonidet P-40 were added. To one-half of each immunoprecipitate, Peptide: N-glycanase F was added. The control reaction was incubated in the absence of N-glycanase F. After incubation at three hours at 37°C, the samples were subjected to SDS-PAGE. The gel was incubated in 25% isopropanol, 10% acetic acid, Amplify (Amersham), dried under vacuum and exposed to film at -70°C with an intensifying screen.

Release of O-fucose glycans by alkali-induced β-elimination and chromatographic analysis—ADAMTS13 radiolabeled with [3H]fucose (from both media and cell lysates) was treated with peptide: N-glycanase F as described above, and the protein was separated from released N-glysans by Sephadex G-50 gel filtration chromatography as described (31). The O-glycans were then released by alkali-induced β-elimination, and the released glycans analyzed chromatographically (gel filtration on a Superdex peptide column, high pH anion exchange chromatography on an MA-1 column) as described (32).

Purification of ADAMTS13 and analysis of tryptic peptides by ion trap mass spectrometry—Conditioned medium from HEK293 cells stably expressing ADAMTS13 (30) was dialyzed against phosphate-buffered saline pH 8.0 and then incubated with Ni-NTA agarose (Qiagen) overnight in phosphate-buffered saline pH 8.0, 4 mM imidazole. ADAMTS13 was eluted from the Ni-NTA agarose with phosphate-buffered saline pH 8.0, 0.25 M imidazole. The purified protein was then dialyzed against Tris-buffered saline pH 7.5. Approximately 1 µg of ADAMTS13 was reduced and alkylated, further purified by SDS-PAGE, subjected to in-gel tryptic digestion, and the resulting tryptic peptides were analyzed by LC-MS/MS using an Agilent XCT ion trap mass spectrometer as described (33). Briefly, peptides were separated by reverse phase capillary HPLC on a Zorbax 300SB-C8 column (3.5 µm diameter beads, 150 X 0.3 mm, Agilent) eluted at 5 µl/min with the following gradient: 0-5 min, 5% B; 5-85 min, 5-35% B; 85-105 min, 35-95% B; 105-115 min, 95% B (A = 0.1% formic acid; B = 95% acetonitrile in 0.1% formic acid). The effluent from the column was sprayed directly into the mass spectrometer under the described conditions (33). Full MS scans (m/z 400-2200) were performed several times each minute, and the two most intense ions in each spectrum were selected for CID fragmentation (MS/MS). Peptides modified with O-fucose glycans were identified by performing neutral-loss searches of the MS/MS data for loss of glucose (hexose, 162 Da), fucose (deoxyhexose, 146 Da) or glucose-fucose (308 Da). Sequential loss of the glucose-fucose disaccharide gives a characteristic fragmentation pattern, allowing easy identification of modified peptides, where the major product ion in the MS/MS spectra is the unglycosylated peptide (see Figure 3). The mass of the unglycosylated peptide was matched to the masses of the predicted tryptic peptides from human ADAMTS13 that contain a predicted O-fucose modification site (C1XX(S/T)C2XXG). Since each of these peptides has a unique mass (see Table 3), there were no ambiguities in assigning the spectra. The presence of b- and/or y-ions (34) in the MS/MS data from fragmentation of the unglycosylated peptide confirmed the assignments. Due to the lability of the glycosidic linkage, assignment of the modified serine or threonine could not be reliably done. Thus, the assignment of the modified residue was based on the consensus sequence. Once glycopeptides...
were identified, additional searches were performed for unglycosylated peptides, or glycopeptides modified with fucose only.

**Generation of O-fucosylation site mutations in ADAMTS13**— Serines at positions 399, 698, 757, 907, 965 were individually changed to alanine by site-directed mutagenesis of the HindIII/EcoRI ADAMTS13 fragment in pBluescript using QuikChange (Stratagene) according to the manufacturer’s directions and the primers listed in Table 1. To generate mutations of serines 1027 and 1087, the EcoRI/XbaI fragment of ADAMTS13 in pBluescript were used in site-directed mutagenesis reactions. The HindIII/EcoRI or EcoRI/XbaI fragments were re-ligated into ADAMTS13 in TOV5. Generation of double mutations was done by site-directed mutation of serine 399 to alanine in the cDNA encoding serine 698 to alanine. Similar double mutations were made with serines 907 and 965, and 1027 and 1087. The coding region of ADAMTS13 was sequenced to verify the serine to alanine codon change and the absence of other mutations.

**Expression of wild-type and mutant proteins**—12 µg wild-type or mutant ADAMTS13 cDNA was transiently transfected into 100 mm dishes of HEK293 TREx cells (Invitrogen) that were approximately 75% confluent using 36 µl Lipofectamine and 24 µl Plus reagent (Invitrogen) according to the manufacturer’s directions in 4 mls of Opti-Mem (Invitrogen). After transfection, the medium was changed to 5 mls Opti-Mem plus 1 µg/ml tetracycline and collected after three days. The cells were washed with phosphate-buffered saline and collected by scraping. The cells were lysed in RIPA buffer as described above. Equivalent fractions of the media and cell lysate were subjected to SDS-PAGE and transferred to Immobilon P (Millipore) by blotting. The blots were incubated with a 1:5000 dilution of peroxidase-conjugated anti-V5 epitope antibody in casein block solution and chemiluminescence was detected with SuperSignal West Pico (Pierce). The percent secretion was calculated after measuring the intensity of the chemiluminescent signal using a Kodak Image Station 440CF. The reported results are the mean of at least three experiments. Statistical significance was analyzed using the Student’s t-test in comparison to the percent of wild-type ADAMTS13 secreted. Results with mutants with a p-value of >0.05 were considered not significantly different from those with wild-type ADAMTS13. Statistical calculations were done using software http://www.graphpad.com/quickcalcs/.

**SiRNA of POFUT2**—RNA oligonucleotides for RNA silencing were created based on the criteria of Ui-Tei et al. with modifications (35). The RNA primers complementary to nucleotides 1135-1153 of POFUT2 were GCACACGCCAGGUUUUUU (sense) and UAAAAACCUGGCGUGGCC (antisense). The control RNA primers directed against luciferase were CUUUACGCUGAGUACUUCGA (sense) and UCGAAGUACUCAGCGUAAG (antisense). The primers were designed with an intentional sense mismatch at the 5-prime antisense base pair. 90 % confluent HEK293 TREx cells in 25cm2 flasks were transfected using 8 µg of the annealed RNA primer pair, and 8 µg of ADAMTS13 or 8 µg of POFUT2 and 20 µl Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. 48 hours after transfection, 1 µg/µl tetracycline was added to de-repress transcription from the tetracycline operon. The medium and cells were collected and analyzed as described 72 hours post-transfection.

**RESULTS**

**O-linked fucosylation of ADAMTS13**— To determine if ADAMTS13 contains O-linked fucose, cells stably-expressing ADAMTS13 were incubated with [3H]fucose for 24 hours, followed by immunoprecipitation of the ADAMTS13 from the cells and medium. Since fucose may exist on proteins as both O-fucose and as a modification of N-glycans, the material was treated with N-glycanase F which cleaves all forms of N-glycans but not O-glycans (Figure 2A). Incorporation of [3H]fucose was seen both in the presence and absence of the N-glycanase
F treatment consistent with incorporation of O-linked fucose (compare lanes 1 and 4 to lanes 2 and 3). The O-fucose glycans were released from the protein by alkali-induced β-elimination after N-glycanase F treatment. Size fractionation of the O-fucose glycans revealed that the majority of the radioactivity is a disaccharide (Figure 2B, data for sample from cells is shown. Similar results were obtained for the samples from media). Very little O-fucose monosaccharide was seen. Analysis of the disaccharide by high pH anion exchange chromatography showed the disaccharide to be Glucose-ß1,3-Fucitol (Figure 2C), the same disaccharide found on TSRs from several other proteins (9-11). Since cellular ADAMTS13 is nearly exclusively present in the endoplasmic reticulum (ER) (27,36), the presence of the Glucose-ß1,3-Fucose disaccharide on cellular ADAMTS13 suggests both the fucose and glucose are added to TSRs in the ER. This is consistent with the reported localizations of both POFUT2 and the β1,3-glucosyltransferase (25,26,37). The increased intensity of the [3H]fucose signal in the medium lane without digestion with N-glycanase F is consistent with the incorporation of fucose in N-linked oligosaccharides as well as in direct O-linkage (compare lanes 3 and 4, Figure 2A).

Interestingly, a small amount of oligosaccharide species was also seen in the analysis of O-fucose glycans (labeled “OS” in Figure 2B). Acid hydrolysis of this material revealed the radiolabel to be mainly in the form of [3H]fucitol (data not shown). Since fucitol is generated during the β-elimination of O-fucose saccharides (but not with fucose on N-glycans), this result suggests the presence of an O-fucose-containing oligosaccharide on ADAMTS13. Further studies will be required to define the structure of this species.

The presence of O-fucose disaccharide at the predicted sites (Figure 1) was analyzed by tandem mass spectrometry. Tryptic peptides were generated from purified human ADAMTS13 and analyzed by LC-MS/MS as described in Experimental Procedures. Fragmentation of peptides modified with the disaccharide resulted in a characteristic pattern showing sequential losses of the glucose (Hex) and fucose (dHex) (Figure 3). The major product ion in the MS/MS spectra corresponds to the unglycosylated peptide. The masses of the major product ions were matched to the predicted masses of tryptic peptides from human ADAMTS13 containing the O-fucose consensus sequence. Tryptic peptides containing six of the seven predicted O-fucosylation sites were identified by this method (within TSRs 2, 3, 5, 6, 7, and 8, see Table 2 and Supplemental Data). The peptide containing the O-fucosylation site from TSR1 was not identified in this analysis. Of the peptides observed, all were found modified with the disaccharide (consistent with the radiolabeling data shown in Figure 2B), except for the peptide from TSR7, where both monosaccharide and disaccharide forms of O-fucose were observed (data not shown).

**Mutation of fucosylation sites can impair secretion of ADAMTS13**—Since the function of O-fucosylation on TSRs is unknown, the effect of O-linked fucosylation on the activity and secretion of ADAMTS13 was characterized. The fucosylated serines were individually mutated to alanines (Figure 1B). Figure 4 shows an example of expression of ADAMTS13 after individual mutation of the fucosylation sites. Unlike other ADAMTS family members, ADAMTS13 is secreted from the cell and is not associated with the plasma membrane or extracellular matrix (27). Typically, about 58% of ADAMTS13 is secreted from the cell after 3 days of incubation. This can be seen in Figure 4A when the secreted ADAMTS13 in lane 8 is compared to the cellular ADAMTS13 in lane 7. TSR1 and TSR3 S to A had very little effect on the secretion of ADAMTS13 from HEK293 cells (compare lanes 1 and 2 and lanes 5 and 6 to lanes 7 and 8) but secretion of TSR2 S to A was dramatically decreased (compare lanes 3 and 4). Secretion of TSR5, TSR6, TSR7, and TSR8 S to A were variably affected as seen in Figure 4B. The secretion of ADAMTS13 and the fucosylation site mutants from multiple experiments was quantified and the results are shown in Table 3. Individual mutation of TSRs 2, 5, 7 and 8 had variable but significant changes in secretion from that of wild-type ADAMTS13. Mutation of fucosylation sites in TSR1, 3, 5, 6, 7, and 8 of ADAMTS13 had no effect on the cleavage of VWF (data not shown). Cleavage of
VWF by TSR2 S to A was unable to be assessed because of the poor secretion of this mutant.

TSR1-6 S to A and TSR1-8 S to A which contain an alanine instead of a serine in TSRs 1,2,3,5, and 6 and in TSRs1,2,3,5,6,7,8 respectively, were generated and expressed in HEK293 cells. Mutation of these multiple sites completely abolished secretion of ADAMTS13 as will be discussed below. Constructs were then made that contained two S to A mutations of TSRs (Figure 5). Mutation of two sites greatly decreased secretion as can be seen with each of these constructs as compared to wild-type ADAMTS13 (compare lanes 4, 6, and 8 to lane 2). Mutation of TSR1+2 S to A essentially abolished secretion of ADAMTS13.

Quantification of these results is reported in Table 3. The double mutants were affected much more than the additive effect of the single mutations. For example, TSR7 and TSR8 S to A individually had a relatively modest effect on secretion but mutation of both residues decreased secretion to 11% as compared to 58% for the wild-type ADAMTS13 protein. The decreased presence of the fucosylation site mutants in the medium is not due to increased degradation since inclusion of protease inhibitors in the medium with tetracycline did not increase the amount in the medium (data not shown).

**Decreased POFUT2 activity impairs secretion of ADAMTS13**—Since the mutation of serines to alanine may impair folding of ADAMTS13 independently of fucose incorporation, fucosylation was blocked in the absence of mutations. Two approaches were used to block fucosylation; first, a CHO cell line, Lec13, that is deficient in GDP-fucose synthesis was utilized (38,39). Lec13 cells are defective in the conversion of GDP-mannose to GDP-fucose but are able to make GDP-fucose if exogenous fucose is added to the medium (38,39). Lec13 cells are defective in the conversion of GDP-mannose to GDP-fucose but are able to make GDP-fucose if exogenous fucose is added to the medium (38,39). Since GDP-fucose is the donor for fucosylation, including that mediated by POFUT2, the Lec13 cells have decreased synthesis of fucose containing oligosaccharides. Figure 6 shows the result when Lec13 cells were transiently transfected with ADAMTS13 or with the D’D3 domains of von Willebrand Factor. D’D3 is normally secreted from cells (28) and does not contain potential O-fucosylation sites. Lec13 cells secrete little or no ADAMTS13 unless they are grown in fucose (compare lanes 2 and 4) but the secretion of D’D3 is unaffected by expression in Lec13 cells grown in the absence (lane 2) as compared to the presence (lane 4) of fucose. Since D’D3 is secreted in the absence of fucose, secretion of protein is not globally affected in the Lec13 cells grown in the absence of fucose. Decreased secretion is not due to reduced fucosylation of N-linked oligosaccharides since ADAMTS13 is secreted normally when processing to a complex-type oligosaccharide is inhibited by the use of deoxymannojirimycin or by expression in GlcNAc I transferase-deficient, Lec1-CHO cells (40) (data not shown).

SiRNA experiments were also performed to decrease the activity of POFUT2 in HEK293 cells. HEK293 cells were transfected with POFUT2 with a Flag epitope tag and POFUT2 siRNA or Luciferase siRNA, as a control. The POFUT2 siRNA but not the control Luciferase siRNA decreased expression of the recombinant POFUT2 by more than 90% as seen in Figure 7A (compare lane 1 and 2) demonstrating the efficacy of the siRNA in decreasing expression levels of POFUT2. Subsequently, HEK293 cells were transiently transfected with ADAMTS13 and with Luciferase siRNA (Luc) or POFUT2 siRNA oligonucleotides, and secretion of ADAMTS13 was examined (Figure 7B). The presence of the POFUT2 siRNA reduced secretion of ADAMTS13 consistent with the importance of O-fucosylation in ADAMTS13 secretion (compare lane 4 to lane 2). A low level of secretion is seen with the siRNA experiments. This is to be expected for several reasons; first the half-life of the endogenous POFUT2, which is approximately 10-12 hours (data not shown) prevents complete depletion of POFUT2 within the duration of the transient-transfection experiment. Also, while the efficiency of POFUT2 silencing is very good, it is not 100%; therefore, some POFUT2 continues to be synthesized and may fucosylate multiple ADAMTS13 molecules during the course of the experiment. Inhibition of ADAMTS13 secretion by both mutation of the fucosylation sites and inhibition of POFUT2 activity is strongly
consistent with the importance of O-fucosylation in proper ADAMTS13 secretion.

Overexpression of POFUT2 promotes TSR1+2 S to A secretion—POFUT1 exhibits a chaperone activity that is distinct from its fucosyltransferase activity since overexpression of fucosyltransferase-inactive POFUT1 promotes the secretion of Notch. To determine if POFUT2 has a chaperone activity in addition to its fucosyltransferase activity, the effect of POFUT2 overexpression on ADAMTS13 secretion was examined. Overexpression of POFUT2 by transient-transfection had no effect on wild-type ADAMTS13 secretion (data not shown). POFUT2 and TSR1+2 S to A were then co-transfected in HEK293 cells. POFUT2 overexpression enabled secretion of TSR1+2 S to A (Figure 8A, compare lane 2 and 4). Based on the fact that wild-type ADAMTS13 secretion is not enhanced by POFUT2 and that significant amounts of unfucosylated peptides of ADAMTS13 were not seen by mass spectral analysis, the enhancement of secretion of TSR1+2 S to A does not appear to be due to increased fucosylation. Thus, it may be due to a chaperone effect of POFUT2. To determine if POFUT2 activity was needed for secretion of TSR1+2 S to A, POFUT2 E395A/E396A was generated. POFUT2 E395 and E396 are within the conserved DXD motif and mutation of the homologous residues in Drosophila OFUT2 eliminated its fucosyltransferase activity (25). Overexpression of POFUT2 E395A/E396A did not enhance secretion of TSR1+2 S to A (Figure 8A, compare lanes 5 and 6 to lanes 3 and 4) but these mutations may have ablated the chaperone activity as well as the fucosyltransferase activity of POFUT2 and prevented the facilitation of secretion. To determine if O-fucose (or O-fucose glycosylation sites) was important for the potential chaperone activity of POFUT2, POFUT2 and TSR1-8 S to A were co-transfected in HEK293 cells. POFUT2 overexpression had no effect on the secretion of TSR1-8 S to A, which lacks all potential fucosylation sites (Figure 8B, compare lanes 5 and 6 to lanes 3 and 4). This suggests that the presence of O-fucose, or of the O-fucose modification sites, may be important for the chaperone activity of POFUT2.

DISCUSSION

Here we demonstrate that human ADAMTS13 is modified with the disaccharide Glucose-ß1,3-Fucose on most of its TSRs. Preventing O-fucosylation by eliminating multiple O-fucose modification sites, reducing cellular GDP-Fucose levels, or reducing POFUT2 using siRNA has a profound effect on secretion of ADAMTS13 from cells. Together these results strongly suggest that O-fucosylation of TSRs plays an important role in processing and secretion of ADAMTS13. Recent results on O-fucosylation of EGF-like repeats within Drosophila Notch suggest that there may be common themes between these two forms of O-fucosylation. Both POFUT1 and POFUT2 are localized to the ER, and both modify properly folded EGF-like repeats and TSRs, respectively (10,22-24). Mutation of O-fucose modification sites causes reduced cell-surface expression of full-length mouse Notch1 (18), and reduced secretion of Drosophila Notch extracellular domain (22). Elimination or reduction of OFUT1 in Drosophila prevents cell-surface expression of full-length Drosophila Notch or secretion of Drosophila Notch extracellular domain (22). Drosophila OFUT1 also shows a chaperone-like activity promoting proper folding of Notch, even when its active site is mutated (22). Here we show that POFUT2 may have a similar chaperone-like activity in addition to its fucosyltransferase activity, in that overexpression of POFUT2 can rescue secretion of TSR1+2 S to A. However, this activity appears to depend on the activity of POFUT2, since an enzymatically inactive mutant POFUT2 does not promote secretion. The presence of O-fucose, or of the O-fucose modification site, appears to be important for the chaperone activity in POFUT2 since POFUT2 overexpression does not enhance secretion of TSR1-8 S to A which lacks O-fucose. It is possible that overexpression of POFUT2 works by increasing the stoichiometry of O-fucosylation on the protein. Further work will define the mechanism of increased secretion with POFUT2 overexpression. Taken together, these results suggest that O-fucosylation of both EGF-like repeats and TSRs plays an important...
role in processing and secretion of proteins containing these motifs.

There are interesting parallels between the role of N-glycosylation in protein folding and O-fucosylation, both of which occur in the ER (22,23). Many glycoproteins require N-linked glycosylation for proper folding and secretion (41) since the oligosaccharide, which is added co-translationally (42,43), can induce a β-turn or prevent premature disulfide bond formation (44,45). Chaperones such as calnexin and calreticulin bind to the N-glycans on newly synthesized proteins in the ER and assist in folding (46). Release from the calnexin/calreticulin folding cycle is mediated by removal of a glucose on the N-glycan modifying the folded protein. Recent studies suggest that the resulting high-mannose type N-glycan is recognized by members of the ERGIC53 family of lectins (47-49). ERGIC53 appears to function as an export receptor, recognizing the N-glycan on a properly folded protein and removing it from the ER (50). O-fucosylation may play a similar role. Since proper disulfide-bonding is required for fucosylation of both EGF-like repeats (24) and TSRs (10), folding of EGF-like repeats and TSR occurs before fucosylation. This suggests that POFUT1 and 2 may provide a signal that an individual EGF-like repeat or TSR is folded. This may be especially important for proteins containing multiple, tandem TSRs or EGF-like repeats (such as ADAMTS13 or Notch) to help the folding machinery keep the cysteines from each motif distinct. Consider that ADAMTS13 has 46 cysteines in its TSRs alone, each of which must find its correct partner before the protein is properly folded. Addition of fucose may serve as a signal, telling the cell that the TSRs or EGF-like repeats are folded, before the protein can leave the ER. The fact that the double mutants generally have significantly larger effects than single mutants (Table 3) suggests that the signal comes from multiple sites, not just one. Lectin-like receptors analogous to ERGIC53 may assist in recognizing properly fucosylated proteins for export. Alternatively, fucosylated proteins may be recognized by a chaperone that facilitates higher order folding; lack of recognition by the chaperone would lead to the poor secretion of the fucosylation double mutants of ADAMTS13.

A third possibility is that fucosylation prevents deleterious interactions that result in decreased efficiency of secretion. Interestingly, C-terminal truncations of ADAMTS13 that do not contain TSR repeats are well secreted (3,27,51). The presence of TSRs and EGF-like repeats in proteins increases the complexity of folding with three disulfide bonds to correctly form in each repeat. Fucosylation may be needed for this complex folding.

It is unclear why fucosylation of different TSRs has such variable effects on secretion, although variable effects are also seen with mutation of the O-fucosylation sites in Notch EGF-like repeats (18). One possibility is that a receptor/chaperone (ERGIC53-like) preferentially recognizes fucose (or glucose-fucose disaccharide) on some TSRs better than others, or may recognize glycans on multiple TSRs. Alternatively, it may be due to the efficiency with which individual TSRs or EGF-like repeats fold. While the sequences of the individual TSRs in ADAMTS13 share a number of common elements (e.g. all but TSR4 contain six conserved cysteines (TSR4 contains 4 of the 6) and all contain a conserved tryptophan), their sequences and lengths vary significantly (2). The most highly conserved sequence (with the exception of TSR4) is the O-fucose modification site (Figure 1B). The fact that serine to alanine mutations within this highly conserved sequence in different TSRs has dramatically different effects on secretion (compare mutation in TSR2 to that in TSR5, 6 or 8, Figure 4) argues that the mutation itself is not causing the effect, but that other sequences within the TSR (where there are differences) are contributing. Presumably, the differences in sequence for each TSR will affect the rate and efficiency of folding for that TSR. The lack of secretion of ADAMTS13 in Lec13 cells grown in the absence of fucose (Figure 6) or when POFUT2 was reduced by siRNA (Figure 7) supports the idea that loss of the fucose modification is causing the effect and not just the mutation. Further experiments will need to be performed to determine whether the efficiency of folding individual TSRs or EGF-like repeats is affected by O-fucosylation.

The fate of the retained fucosylation site mutants is also unclear. The half-life of cellular
wild-type ADAMTS13 is approximately seven hours with most of the protein being secreted (36). Using pulse-chase experiments, TSR1+2 S to A is detected within the cell even after 24 hours of incubation without secretion (data not shown). It is unclear if intracellular efforts are made to promote secretion of cellular TSR1+2 S to A instead of targeting it for rapid degradation.

TSRs 1,2,3,5,6,7, and 8 of ADAMTS13 all contain the originally described consensus sequence for O-fucosylation of TSRs of CSX(S/T)CG (9) (Figure 1B). These sequences do not completely match the revised consensus sequence of WX\(\_2\)C\(\_1\)X\(\_2/3\)(S/T)C\(\_2\)X\(\_2\)G (11) since they contain six or seven amino acids between the W and the C\(\_1\) instead of five. Further work will need to be done to more precisely define the role of the tryptophan in the consensus sequence for O-fucosylation. Except for the spacing after the tryptophan, the consensus sequence is conserved in fish (Fugu rubripes) and mammals (Mus musculus and Homo sapiens), in ADAMTS13 TSRs 1,2,3,5,6,7, and 8 suggesting an evolutionary importance of O-fucosylation for this protein. This is consistent with our results demonstrating the importance of O-fucosylation for proper secretion of ADAMTS13.
REFERENCES


Acknowledgements—We thank Dr. Stuart Kornfeld (Washington University) for critical reading of this manuscript. We would also like to thank Dr. Anne Dell for helpful suggestions regarding the mass spectral data.
FOOTNOTES

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1 The abbreviations used are: TSR, thrombospondin type I repeat; POFUT2, protein O-fucosyltransferase 2; POFUT1, protein O-fucosyltransferase 1; ER, endoplasmic reticulum; EGF, epidermal growth factor; CHO, Chinese hamster ovary; Hex, hexose, dHex, deoxyhexose.

FIGURE LEGENDS

Figure 1. Domain structure of human ADAMTS13. A. Domain structure of mature ADAMTS13 consists of a metalloprotease domain (MP), a disintegrin domain (Dis), a TSR, a Cys-rich domain, a spacer domain, seven additional TSRs and two CUB domains. Potential O-fucosylation sites within the TSRs are indicated with a *. B. Amino acids surrounding the potential O-fucosylation site (*) within the TSRs matching the original consensus sequence: C1XX(S/T)C2G (9).

Figure 2. ADAMTS13 is modified with the O-fucose disaccharide Glucose-ß1,3-Fucose. A. HEK293 TREx cells stably-transfected with ADAMTS13 were labeled with [3H]fucose for 24 hours. Samples of cell lysate (C) and media (M) were immunoprecipitated with anti-V5 and treated without (-) or with (+) Peptide: N-glycanase F. Products were analyzed by SDS-PAGE and fluorography. Molecular mass standards are indicated at the left. B. Gel filtration chromatography on a Superdex peptide column of [3H]fucose-labeled glycans released from ADAMTS13 (purified from cell lysates) by alkali-induced β-elimination. Migration positions are indicated for monosaccharides (Mono), disaccharides (Di), trisaccharides (Tri), and oligosaccharides (OS). A similar profile was obtained for ADAMTS13 isolated from medium. C. High pH anion exchange chromatography of the disaccharide species from panel B. Elution positions of standards is indicated: β1,2 is Glucose-ß1,2-Fucitol; β1,3 is Glucose-ß1,3-Fucitol; β1,4 is Glucose-ß1,4-Fucitol.

Figure 3. TSR2 is modified with O-fucose disaccharide. Tryptic peptides from human ADAMTS13 were analyzed by LC-MS/MS as described in Experimental Procedures. Searches of the MS/MS data for neutral loss of 154 (loss of the glucose-fucose disaccharide from a doubly charged peptide) revealed the spectra shown. Top panel: MS spectrum showing the major ions present at 25.5 minutes of the HPLC run. The mass spectrometer selects the two most abundant ions for CID fragmentation in the next two cycles. Ions selected for fragmentation in each MS scan are identified by diamonds. M corresponds to the monoisotopic mass for the peptide plus dHex and Hex. One of the selected ions corresponds to the doubly charged form of this glycopeptide ([M+2H]^{2+}, m/z 765.0). The other ions present in the MS spectrum (e.g. m/z 636.5) represent other peptides eluting from the reverse phase HPLC column at the same time. Bottom panel: MS/MS spectrum of the m/z 765.0 ion from the top panel. The location of the parent ion ([M+2H]^{2+}, m/z 765.4) prior to fragmentation is indicated in the MS/MS spectrum with a diamond. Ions corresponding to the sequential losses of a hexose ([M+2H-Hex]^{2+} and a deoxyhexose ([M+2H-Hex-dHex]^{2+}) from the parent ion are indicated. The major product ion ([M+2H-Hex-dHex]^{2+}, m/z 611.1) corresponds to the doubly charged form of GPCSVSCGAGLR704, a tryptic peptide from TSR2 that contains an O-fucose consensus sequence (see Table 2 for predicted masses). No other predicted tryptic peptides from human ADAMTS13 correspond to this mass. Further confirmation for the assignment of the peptide comes from peptide fragment ions. Several y-ions from fragmentation of the peptide are shown here. The spectra for the other glycopeptides reported in Table 2 are shown in the...
Supplemental Data. The small amount of m/z 611.1 in the MS spectra probably corresponds to glycopeptide that lost the saccharide during the ionization process (in-source decay) (52). O-Fucose is known to be partially lost from glycopeptides during ionization, but O-fucosylated peptides are known to separate from unglycosylated peptides during reverse phase HPLC (52). Thus, unglycosylated peptide should not appear in the same spectrum as glycopeptide. Additional searches confirm that no unglycosylated peptide can be found in MS spectra at later times in the HPLC run.

Figure 4. TSR2 S to A, TSR5 S to A, and TSR7 S to A impair secretion of ADAMTS13. HEK293 TREx cells were transiently-transfected with wild-type ADAMTS13, TSR1 S to A, TSR2 S to A, or TSR3 S to A, TSR5 S to A, TSR6 S to A, TSR7 S to A and TSR 8 S to A. Equivalent fractions of cell lysates (C) and media (M) were subjected to SDS-PAGE and analyzed by Western blotting with anti-V5 antibody. A. TSR1, TSR2, TSR3 S to A changes and wild-type ADAMTS13. B. TSR5, TSR6, TSR7, and TSR8 S to A changes.

Figure 5. Mutation of more than one TSR fucosylation site nearly abrogates secretion of ADAMTS13. HEK293 TREx cells were transiently-transfected with wild-type ADAMTS13, TSR1+2 S to A, TSR5+6 S to A, or TSR7+8 S to A. Equivalent fractions of cell lysates (C) and media (M) were analyzed by SDS-PAGE and Western blotting with the anti-V5 antibody. Molecular mass standards are indicated at the left.

Figure 6. Impairment of TSR fucosylation decreases secretion of ADAMTS13. Lec13 cells grown in the presence (+) or absence of fucose (-) were transiently-transfected with ADAMTS13 or with the D’D3 domains of VWF. Equivalent fractions of cell lysates (C) and media (M) were analyzed by SDS-PAGE and Western blotting with the anti-V5 antibody to detect ADAMTS13 or with anti-VWF antibody to detect D’D3.

Figure 7. Decreased expression of POFUT2 decreases ADAMTS13 secretion. A. Carboxyl-Flag epitope tagged POFUT2 and control Luciferase (Luc) oligonucleotides or carboxyl-Flag epitope tagged POFUT2 and POFUT2 siRNA oligonucleotides were transiently-transfected in HEK293 TREx cells. Equivalent fractions of the medium were analyzed by SDS-PAGE and Western blotting with an anti-Flag antibody. B. HEK293 TREx cells were transiently-transfected with ADAMTS13 and control Luciferase (Luc) or with ADAMTS13 and POFUT2 siRNA oligonucleotides. Equivalent aliquots of cell lysates (C) and media (M) were analyzed by SDS-PAGE followed by Western blotting with anti-V5 antibody.

Figure 8. POFUT2 improves secretion of TSR1+2 S to A but not TSR1-8 S to A. A. HEK293 TREx cells were transiently-transfected with TSR1+2 S to A and with or without carboxyl- Flag POFUT2 or fucosyltransferase dead carboxyl- Flag POFUT2, E395A/E396A. Equivalent fractions of cell lysates (C) and media (M) were subjected to SDS-PAGE and analyzed by Western blotting with anti-V5 antibody. B. HEK293 TREx cells were transiently-transfected with wild-type ADAMTS13 or with TSR1-8 S to A alone or with TSR1-8 S to A and carboxyl- Flag POFUT2. Equivalent fractions of the cell lysates and media were analyzed by SDS-PAGE and Western blotting with anti-V5 antibody. Molecular mass standards are indicated at the left.
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<th>Primer Set</th>
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<tr>
<td>S to A 399</td>
<td>5'-ccttgctccccgcgctgagaggagtgg-3’</td>
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**Amino-Flag**

**POFUT2**

5’-gactacaaagacgatgacgataagggccaggagttctggcccggacatcgg-3’
5’-tcagtaggtgatctccagtg-3’

**Carboxyl-Flag**

**POFUT2**

5’-ccgggcccagcctggttcggtcggagaggtgtgcgtg-3’
5’-tcacttatgtgtgatgtgtgcgtg-3’

**Signal Sequence**

5’-agttgcaccacattgagaagacagcactgctctgtgatgtgtgtatgtgtgcgtg-3’
5’-agttgcaccacattgagaagacagcactgctctgtgatgtgtgcgtg-3’

**EcoRI**

**ADAMTS13**

5’-gggcaaaaacagaccaatctctccgcaagc-3’
5’-gggtgtgcggcagaagctttcgtgctg-3’

**POFUT2**

5’-cctttgcagatctgctgagaggtgtgcgtg-3’
5’-ccaggtgattttctctccgcaagc-3’

**Table 1.** Primers used to generate mutations, POFUT2, and pSignal Sequence.
Glycopeptides identified from human ADAMTS13:

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<td>1724.6</td>
<td>1723.8</td>
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<td>2574.4</td>
<td>2266.3</td>
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<td>308.1</td>
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Table 2: *O*-fucose modified peptides identified from ADAMTS13. The predicted *O*-fucose modification site, based on the consensus sequence C1XX(S/T)C2XXG, is underlined in the tryptic peptides. Singly charged masses were calculated from the parent ions for each spectra (see Figure 3 or Supplemental Data). Charge state was determined based on the charge state of peptides as determined from the neutral losses of glucose (Hex) and fucose (dHex). “[M+H]$^+$ of parent” refers to singly charged mass of the parent ions. “[M+H]$^+$ of product” refers to singly charged mass of the major product ion in each spectra, which corresponds to the unglycosylated peptide. “Predicted [M+H]$^+$” was calculated using monoisotopic masses for the carboxyamidomethylated peptides < 2000, but average masses for peptides > 2000. “Parent – product” shows the difference between the singly charged parent and major product ions, which matches the predicted mass of the glucose-fucose disaccharide (308.1).

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Table 3. Quantification of secretion data from Figures 4 and 5. HEK293 TReX cells were transiently-transfected with the indicated constructs. Equivalent fractions of cell lysates and media were analyzed by SDS-PAGE and Western blotting with anti-V5 antibody. The intensity of the signals was measured and the percent in the cell lysate and medium was determined. “n” is the number of independent experiments performed. SD represents the standard deviation of the percent of the mutant or wild-type ADAMTS13 secreted and was calculated using http://www.graphpad.com/quickcalcs/Clmean1.cfm. P-values were determined using the Student t-test in comparison to wild-type ADAMTS13 secretion as calculated using http://www.graphpad.com/quickcalcs/ttest1.cfm. P-values greater than 0.05 were considered not statistically different from wild-type (NS.)
A.

![Diagram of protein structure with annotations TSR1, TSR2, CUB 1-2, MP, Dis, Spacers, and Cys-Rich regions.]

B.

```
CSRSCG  TSR1
CSRSCG  TSR2
CSASCSCG TSR3
CTSAGG  TSR4
CSVSCG  TSR5
CSVSCG  TSR6
CSVSCG  TSR7
CSVSCG  TSR8
```
Figure 2
Figure 3

$^{693}_{693}$GPCSV$^{704}_{704}$SCGAGLR$^{704}_{704}$ + dHex-Hex (2+)

$m/z = 765.0$
A. 

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B. 

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9  10  11  12  13  14  15  16

Figure 4
Figure 7
A. TSR 1+2 S to A

- - + + - - POFUT2
- - - - - + + POFUT2 EEAA
C M C M C M

1 2 3 4 5 6

kDa

190 131 93

B. ADAMTS13 TSR1-8 S to A

- - - - - + + POFUT2
C M C M C M

1 2 3 4 5 6

kDa

190 131 92

Figure 8
O-Fucosylation is required for ADAMTS13 secretion
Lindsay M. Ricketts, Malgosia Dlugosz, Kelvin B. Luther, Robert S. Haltiwanger and Elaine M. Majerus

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