Identification of Functional Glycosylation of ADAMTSL2

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Abstract of the Thesis

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ADAMTSL2 has been shown to play a role in regulation of Transforming Growth Factor β (TGFβ) signaling through binding Latent TGFβ Binding Protein 1 (LTBP1) and Fibrillin 1 (FBN1) in the extracellular matrix. A genetic screen revealed mutations to Adamtsl2 cause a rare growth disorder called Geleophysic Dysplasia (GD). Multiple GD mutations fall within ADAMTSL2’s seven Thrombospondin Type 1 Repeats (TSRs). TSRs often undergo a form of glycosylation called O-fucosylation. The addition of a fucose sugar to TSRs has been shown to be a necessary process in the secretion of related proteins. Among several mutations to ADAMTSL2 within TSRs which cause GD, two are predicted to interfere with O-fucosylation. In this study we reproduced these two GD-associated mutations as well as three additional mutations predicted to interfere with glycosylation in an unusual O-fucosylation site on TSR6. The predicted O-fucose site on TSR6 actually overlaps with a predicted site of N-glycosylation. Our three TSR6 mutant constructs were designed to both address which type of modification is occurring as well as its importance in protein secretion. We utilized expression constructs incorporating these mutations in parallel transactions to assay their effect on protein secretion in 293T cells. We predict that mutations predicted to affect O-fucosylation will impair secretion, while our mutation interfering with N-glycosylation will not. In this way we hope to provide a functional link between mutations to ADAMTSL2 and GD.
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<tr>
<td>AD</td>
<td>Acromicric Dysplasia</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloprotease with thrombospondin Type 1 Repeats</td>
</tr>
<tr>
<td>B13GTL</td>
<td>Beta-1,3,-Glucosyltransferase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CEL</td>
<td>Camurati-Engelmann Disease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FBN1</td>
<td>Fibrillin-1</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>hIgG</td>
<td>Human Immunoglobulin G</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparin Sulfate Proteoglycan</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency Associated Peptide</td>
</tr>
<tr>
<td>LLC</td>
<td>Large Latent Complex</td>
</tr>
<tr>
<td>LTBP1</td>
<td>Latent TGFβ Binding Protein 1</td>
</tr>
<tr>
<td>MS</td>
<td>Myhre Syndrome</td>
</tr>
<tr>
<td>OST</td>
<td>Oligosaccharyl Transferase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyylimine</td>
</tr>
<tr>
<td>POFUT2</td>
<td>Protein O-fucosyl Transferase 2</td>
</tr>
<tr>
<td>pSMAD</td>
<td>Phospho-smad-Mothers Against Decapentaplegic</td>
</tr>
<tr>
<td>SLC</td>
<td>Small Latent Complex</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TSR</td>
<td>Thrombospondin Type 1 Repeat</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>vWF</td>
<td>Von-Willebrand Factor</td>
</tr>
<tr>
<td>WMS</td>
<td>Weil-Marchesani Syndrome</td>
</tr>
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Introduction

Geleophyic Dysplasia (GD) is a rare autosomal recessive growth disorder which can occur as a result of mutations to A disintegrin and metalloproteinase with thrombospondin repeats-like 2 (Adamtsl2) (Allali, Le Goff et al. 2011; Le Goff, Mahaut et al. 2011). The GD phenotype is characterized by upturned corners of the mouth (hence the Greek etiology Geleo-, meaning “happy”), hepatomegaly, shortened tubular bones, delayed bone growth, short stature, and severe thickening of skin and heart valves (Le Goff, Morice-Picard et al. 2008). GD is a form of acromelic dysplasias, a family of four disorders which share similar clinical manifestations (Allali, Le Goff et al. 2011). Other types of acromelic dysplasia include acromicric dysplasia (AD), Wiel-Marchesni Syndrome (WMS), and Myhre Syndrome (MS). All of which differ subtly in phenotype and pattern of inheritance (Le Goff C 2012) (Figure 1 A, B). GD is the most severe acromelic dysplasia in that patients exhibit bronchiopulmonary insufficiency, tracheal stenosis, and progressive thickening of cardiac valves which is the primary cause of early death, often before five years of age(Pontz BF 1996). These clinical features correlate well with areas of high ADAMTSL2 expression and can be attributed to aberrant TGFβ signaling (Le Goff, Morice-Picard et al. 2008)(Figure 1, C).

ADAMTS protein family

ADAMTSL2 is a member of the Disintegrin and Metalloproteinase with Thrombospondin Repeats, ADAMTS) superfamily of metalloproteases. This family consists of nineteen zinc metalloproteases with a wide assortment of substrates
including delta I, procollagen II, aggrecan, versican and large vWF (Fernandes, Hirohata et al. 2001; Levy, Nichols et al. 2001; Sandy JD 2001; Zheng, Chung et al. 2001; Six E 2003). In addition to the nineteen ADAMTS proteins there are seven ADAMTS-like proteins which share the same characteristic structure but lack a catalytic protease domain (Apte 2009). ADAMTS-like proteins, like their metalloprotease cousins, typically have a variable number of thrombospondin type 1 repeats, (TSRs) on the C-terminal end (Apte 2009).

**Adamtsl2** was first identified in a large scale screen to find genes expressed in the human brain (Nagase T 1998). The gene structure was then annotated by comparative sequence analysis of the mouse and human genomes as well as ESTs (Koo BH 2007). *Adamtsl2* is typically expressed in liver, kidney, heart, brain, skeletal muscle, placenta, and lungs (Koo BH 2007). The expression pattern of *Adamtsl2* during mouse development infers a significant function in organogenesis and especially differentiation of immature skeletal muscle myoblasts (Koo BH 2007). *In situ* hybridization of human fetus at thirty five weeks of gestation showed dense areas of *ADAMTSL2* expression in the tracheal wall, cardiomyocytes, epidermis, dermal blood vessels, and pulmonary arteries (Le Goff, Morice-Picard et al. 2008). The ADAMTSL2 protein is characterized by the presence of an N-glycan rich module, and seven TSRs. The presence of thrombospondin type 1 repeats (TSRs) and an N-terminal signal peptide allowed researchers to infer that ADAMTSL2, like other members of the ADAMTS superfamily, is a secreted glycoprotein. This was subsequently verified by immunostaining transfected CHO and HEK293F; these results demonstrated that ADAMTSL2 is secreted to the extracellular matrix or cell media, respectively, depending
on a cell’s ability to accumulate extracellular matrix (Koo BH 2007). Later studies demonstrated that GD mutations to \textit{ADAMTSL2} caused significantly reduced secretion and linked this impairment to increased TGFβ signaling (Le Goff, Morice-Picard et al. 2008).

\textit{ADAMTSL2 in signaling}

To determine the functional significance of GD mutations to \textit{ADAMTSL2} expression constructs were designed to assay secretion, and found that these mutations cause significant defects in secretion without significant cellular accumulation of protein, meaning that GD mutations cause defective protein processing (Le Goff, Morice-Picard et al. 2008) in addition to defects in secretion of ADAMTSL2, fibroblasts from GD patients exhibited significantly higher levels of active TGFβ in the media as well as nuclear pSMAD2, indicating a loss of TGFβ inhibition (Le Goff, Morice-Picard et al. 2008; Le Goff, Mahaut et al. 2011). In order to deduce where ADAMTSL2 fits in with respect to TGFβ regulation a yeast two hybrid screen was performed identifying LTBP1 as a binding partner, which was later confirmed by coIP (Le Goff, Morice-Picard et al. 2008). ADAMTSL2 was also shown to bind directly to fibrillin-1 (FBN1) by surface plasma resonance in an investigation of FBN1’s functional significance in GD patients without mutations in adamtsl2 (representing 57.6% of screened GD patients) (Le Goff, Mahaut et al. 2011). Thus the enhanced TGFβ signaling in GD patients likely results from disorganization of the microfibril network in GD patient fibroblasts resulting in an increase in the bioavailability of TGFβ. Given the role of ADAMTSL2 in this regulation it is critical that this protein be processed and secreted efficiently for proper development.
ADAMTSL2 goes through a complex series of post-translational modifications in order to facilitate proper folding and eventual secretion. In most cases, TSRs are modified with an O-linked fucose carbohydrate in the ER by Protein O-fucosyltransferase 2 (POFUT2) that is further elongated to fucose-glucose disacharride in the medial golgi by β-1,3 glucosyltransferase (B13GTL) (Hofsteenge, Huwiler et al. 2001; Shao L 2003; Kozma K 2006). O-fucosylation is necessary for proper protein folding and secretion of thrombospondin 1 and other TSR containing proteins (Ricketts LM 2007; Wang 2007; Luther KB 2009).

Structural Considerations and interactions of Thrombospondin Repeats

A major feature of ADAMTSL2 is seven TSR domains with a characteristic folding pattern. Most TSRs on ADAMTSL2 carry a conserved consensus sequence for O-fucosylation, a special form of glycosylation occurring in the endoplasmic reticulum by POFUT2 as well as a consensus for C-mannosylation, WXXWXXW, by a currently unknown C-mannosyltransferase (Luo, Koles et al. 2006). Using GDP-fucose as a substrate POFUT2 catalyzes the transfer of a fucose sugar to the consensus sequence CX_{2-3}(S/T)X_{2}G of properly folded TSRs (Hofsteenge, Huwiler et al. 2001; Luo, Koles et al. 2006; Wang 2007; Chen, Keusch et al. 2012). The O-fucose consensus sequence forms a flexible loop between N-terminal end and the first β-ribbon, called the AB loop (Tan, Duquette et al. 2002). The TSR carries six conserved cysteine residues forming three disulfide bonds, two β-ribbons, and a “jar handle” forming a right handed twist capable of interacting with various coagulation factors such as vWF and integrins (Chen, Herndon et al. 2000; Tan, Duquette et al. 2002). Recent crystallographic
analysis of TSR2 & TSR3 from human thrombospondin 1 and drosophila F-spondin was completed to characterize the important structural motifs for TSR interaction with POFUT2. During this course of study it was also shown that any mutations which affect the disulfide bonding pattern or structural integrity of the AB loop significantly reduce fucosylation (Chen, Keusch et al. 2012). Since only properly folded TSRs can be fucosylated it is reasonable to infer that POFUT2 functions to validate proper folding of TSRs before trafficking to the medial golgi, similar to other forms of glycosylation (Roth, Zuber et al. 2010). In this study we hope to elucidate the importance of O-fucosylation in ADAMTS2 by mutating the O-fucose site on TSR6 as well as produce GD associated mutations and assay protein secretion.

RESULTS

*Site Directed Mutagenesis and Sequence Confirmation*

A previous study by Le Goff, et al., showed that geleophysic dyplasia mutations in *ADAMTS2* impaired protein secretion (Le Goff, Morice-Picard et al. 2008). One of the mutations they assayed, G811R, fell within the CX_2-3(S/T)CX_2G O-fucosylation consensus sequence in TSR6 (Le Goff, Morice-Picard et al. 2008). In a second genetic screen of 33 GD patients, 17 previously undocumented mutations to *ADAMTS2* were identified. One of these mutations S635L, was located at the X_2 position in the TSR3 O-fucosylation consensus (Allali S 2011). It is not known whether this mutation similarly impairs secretion of ADAMTS2. O-fucosylation of TSRs is critical for processing and secretion of ADAMTS13 and ADAMTS1 in cell culture (Ricketts LM 2007; Wang, Dlugosz et al. 2007). Based on these observations, we predict that O-fucosylation is
similarly critical for processing and secretion of ADAMTSL2. A major goal of this project is to determine whether GD-like mutations impair mouse ADAMTSL2 secretion as a result of defects in glycosylation.

As a first step towards this goal, we generated myc-tagged *Adamsl2* expression constructs carrying mutations that result in substitutions in TSR3 (S641L) or TSR6 (G817R) that are similar to the human GD mutations S635L and G811R, respectively. In addition, we generated mutations that disrupt predicted sites of *O*-fucosylation and *N*-glycosylation in ADAMTSL2 TSR6 (N813Q, T815V, and N813Q;T815V). TSR6 is unusual in that a consensus for *N*-glycan addition (NXS/T) is embedded within the *O*-fucosylation consensus sequence CX2-3(S/T)CX2G. The N813Q mutation is predicted to disrupt *N*-glycosylation. Whereas, the T815V, and N813Q;T815V mutations are predicted to disturb both glycosylation and fucosylation.

Dr. Suneel Apte’s group of the Lerner Research Institute in Cleveland, Ohio generated a *Wild-type Adamsl2* plasmid mutants(Koo, Le Goff et al. 2007). We transferred the *Adamsl2* sequence into the pSecTag2B hygroC expression vector, and used this clone as a template for PCR based site directed mutagenesis generation of *Adamsl2* mutants using primers listed in Table 1 (Materials and Methods). Geleophysic Dysplasia-like mutant construct G817R and TSR6 mutants N813Q, T815V, and N813Q;T815V were originally constructed by Sharee Sandler in the Haltiwanger/Holdener labs. I performed site directed mutagenesis generate ADAMTSL2 S641L, using primers originally designed by Sharee Sandler (Table 1.; Materials and Methods).
Before beginning *in vitro* expression assays using these constructs, I submitted all clones for re-sequencing to confirm mutations. Analysis of the sequence suggested that the T815V expression construct DNA prep was contaminated by the N813Q;T815V double mutant plasmid, and that the N813Q;T815V expression construct DNA prep was contaminated with T815V plasmid. To resolve this issue I struck out single colonies from one transformant from both questionable clones and sequenced plasmid DNA from five ‘second generation’ single colonies to isolate uncontaminated plasmid stocks for each clone (Materials & Methods). In this way, I identified single colony isolates which carried the intended mutation(s). I used these isolates for large-scale preparation of plasmid DNA, and submitted DNA samples with primers listed in Table 2 to the Stony Brook University DNA sequencing facility.

I compared the sequence of the wild-type and mutant *Adamtsl2* clones using Sequencher 5.0 software. The names and dates of original sequence files and Sequencher contigs are listed in Table 3. DNA sequences for each clone covered the entire *Adamtsl2* insert as well as flanking regions of the pSecTag2B vector. In Figure 3, the *Wild-type* and mutant clone sequences are aligned in the region surrounding the mutations. Outside these regions, the mutant clone sequences were identical to the parent *Adamtsl2* pSecTag2B clone. However we detected an unexpected single nucleotide substitution at nucleotide position 903 (relative to the start Met) compared to the reference sequence in the Genbank database (NM_029981.1) (data not shown) present in all clones. This mutation results in a valine to isoleucine substitution (V897L). This nucleotide substitution was also detected in the original plasmid provided by S. Aptee (data not shown). Although V897 is a highly conserved residue, V897L is
not predicted to have an effect on protein function, folding or secretion due to the similar biochemical nature of these two amino acids.

*Mutations to O-fucose site in TSR6 impair protein secretion*

To begin to address whether glycosylation of ADAMTSL2 TSR6 was needed for protein maturation, we looked at the effects of the N813Q, T815V, and N813Q;T815V mutations on ADAMTSL2 secretion. The consensus sequence for O-fucosylation, CX_{2-3}(S/T)CX_{2-3}G, in TSR6 is unusual in that it overlaps an N-glycan site, NX(S/T), recognized by oligosaccharyl transferase (OST) (Figure 4 A). The T815V mutant eliminates the essential modified threonine residue in the O-fucosylation consensus sequence CX_{2-3}(S/T)CX_{2-3}G. However, since the threonine residue is also part of the conserved N-glycosylation consensus, NX(S/T), we predict that this mutation will block both N-glycosylation as well as O-fucosylation. This mutation reduced ADAMTSL2 secretion to 20.0% (±12.1%) of Wild-type (Figure 4 C). Secretion of ADAMTSL2 carrying both the N813Q and T815V mutations significantly reduced to 3.6% (±2.1%) of WT (Figure 4 C). The N813Q mutant disrupts the conserved asparaginine in the consensus recognized by OST, and is predicted to block N-linked glycosylation. Since N-glycosylation occurs co-translationally and is predicted to prevent O-fucosylation of the neighboring S/T residue recognized by POFUT2, this mutation may actually facilitate O-fucosylation of TSR6. Assuming O-fucosylation is present on TSR6 and promotes secretion, I predicted this change would not interfere with secretion of ADAMTSL2. Contrary to my prediction, the N813Q mutation reduced secretion of ADAMTSL2 protein to 14.7% (±5.9%) of WT (Figure 4 C). This data also shows that the N813 is more functionally significant than T815, this contradicts our prediction that the modified residue is the most important for
secretion. Although secretion was impaired we did not observe cellular retention (or buildup) of protein in all three cases (or in any case)(Figure 4C). On the contrary we see a large reduction in cellular protein when the N-glycosylation site is disrupted, suggesting rapid degradation. Taken together, these data demonstrate that mutations predicted to interfere with TSR6 N-glycosylation, not O-fucosylation, site significantly impair protein secretion. This data also suggests that N-glycosylation by OST is more likely to occur at this site. Future studies will utilize mass-spectral analysis to ascertain the effect of these mutations on glycosylation, thus providing a functional link between glycosylation and secretion.

**Geleophysic Dysplasia Mutations to Adamtsl2 cause impaired secretion**

Given that the mutations disrupting TSR6 glycosylation all lead to decreased secretion it is reasonable to predict that GD-like mutations that fall within these consensus sequences may also interfere with glycosylation and similarly impair protein trafficking. Both GD-like substitutions, S641L and G817R occur at positions within the O-fucose consensus occur in positions predicted to tolerate variability, so called “X” positions. Using myc tagged Adamtsl2 expression plasmids we assayed secretion of GD-like mutations G817R and S641L relative to WT. Based on previous analysis of the human ADAMTSL2 G811R by Le Goff et al we predicted that the mouse G817R and S641L mutations would lead to significantly decreased secretion(Le Goff, Morice-Picard et al. 2008). Consistent with this prediction, the S641L and G817R mutations reduced ADAMTSL2 secretion to 27.8% (± 9.83%) and 9.5% (±2.63%) of WT, respectively (Figure 5 C). No significant increase in intracellular ADAMTSL2 protein was observed (Figure 5 C). To determine whether the secretion defects result from impaired
glycosylation, future experiments will use mass-spectral approaches to determine the glycosylation status of these TSR loops.

**Discussion**

Western Blot analysis of protein levels in the conditioned media showed that all assayed substitutions lead to a decrease in secretion. The decrease was observed regardless of what position within the O-fucose consensus was mutated. However, the degree in which secretion was affected varied from mutant to mutant. Although we are able to make a statement on the functional consequence of mutation we do not yet know what effect most of these mutations have on the efficiency of fucosylation. During the course of our experiments a recent publication revealed mutations affecting structure of TSRs causes decreased fucosylation efficiency (Chen, Keusch et al. 2012). The variable effect of these mutations on secretion may be indicative of causative defects in efficiency of fucosylation. And this deficiency in fucosylation may be related to structural effects mutations to the O-fucosylation sequon.

*Secretion is necessary for ADAMTSL2 mediated regulation of TGFβ signaling*

TGFβ ligand is synthesized and secreted as an inactive propeptide homodimer in the so called small latent complex (SLC) which is then subsequently bound with latent TGFβ binding protein 1 (LTBP1) in the large latent complex (LLC) (Mangasser-Stephan K 1999; Rifkin 2005). Interactions between LTBP1s (and subsequently the LLC), heparin sulfate proteoglycans (HSPGs), and fibronectin initially occur in the pericellular space (Kantola AK 2008). When necessary, activation occurs during myofibroblast contraction
via αvβ6 integrin binding to LLC via LTBP1 (Wipff, Rifkin et al. 2007). Fibroblasts derived from GD patients exhibiting mutations to ADAMTSL2 displayed a significant increase in TGFβ-Smad signaling (Le Goff, Morice-Picard et al. 2008). ADAMTSL2 acts to regulate this signaling through direct interaction with Latent TGFβ binding protein 1 (LTBP1), which is known to regulate availability of mature TGFβ in the ECM (Koo, Le Goff et al. 2007; Le Goff C 2012) (Figure 1, C). Defective regulation of TGFβ in the ECM, either by mutations to ADAMTSL2 or TGFβ associated FIBRILLIN-1, causes aberrations in microfibril organization of skin fibroblasts which in turn causes the thick skinned phenotype (Figure 1, A & B) characteristic of GD and related disorders (Le Goff, Morice-Picard et al. 2008; Le Goff, Mahaut et al. 2011). The aberrations in microfibrillar network organization likely give rise to the range of phenotypes due to defective sequestering of TGFβ via LTBP1 and FBN1 (Ramirez and Rifkin 2009). This is consistent with studies linking LTBP1 deficiency with cardiac valvular thickening (Ramirez and Rifkin 2009; Todorovic V 2011). Thusly these clones can be used to further validate the importance of O-fucosylation in the pathogenesis of disorders like GD.

*Structural Consequences of Mutations to O-fucose site*

POFUT2 typically will only recognize and fucosylate properly folded TSRs (Luo, Nita-Lazar et al. 2006). This, like other forms of glycosylation, may serve as a quality control mechanism verifying proper protein folding (Maattanen, Gehring et al. 2010). Homozygous mutations in Adamtsl2 have been linked to GD, however the structural implication of these mutations has not been specifically characterized in most cases.
TSRs typically consist of approx 60 amino acids, including six conserved cysteine residues begetting 3 disulfide bonds, and consensus sequences for C-mannosylation and O-fucosylation (Adams and Tucker 2000). ADAMTSL2 has seven of these TSRs as well as N-glycan rich module, which makes it a unique member of the ADAMTS superfamily (Koo, Le Goff et al. 2007; Apte 2009). ADAMTSL2 has multiple TSRs, each of which must be folded properly to facilitate secretion, presents a challenge for the cell’s protein processing machinery. N-linked glycosylation and O-fucosylation are utilized by the cell to provide another level of regulation to ensure the proper protein folding, which is necessary for TSR containing proteins like ADAMTSL2 (Shao L 2003; Luther KB 2009). The organization of TSR secondary structure forms a stable left-handed face necessary for interaction with cell surface receptors and structural components of the ECM (Tan, Duquette et al. 2002; Apte 2009). The focus of my thesis work was on the importance of the O-fucosylation consensus motif, CX_{2,3}(S/T)CX_{2}G. Although the primary sequence is important as a substrate for POFUT2, the structural features of the TSR play an equally important role in efficiency of fucosylation and/or secretion. The CX_{2,3}(S/T)CX_{2}G motif was shown to form the flexible AB loop downstream of a C-mannose site, characterized by 3 conserved tryptophan residues (Gonzalez de Peredo 2001; Tan, Duquette et al. 2002). The shape of the AB loop is such that it fits into a small pit within the central cavity of POFUT2 thereby allowing transfer of a fucose sugar to the central serine or threonine within the O-fucosylation consensus sequence (Chen, Keusch et al. 2012). Recent crystallographic studies using mutant TSR4 from drosophila F-Spondin has shown that eliminating the disulfide bond closest to the AB loop by mutagenesis of cysteine or increasing the size of the loop by
introducing an extra glycine residue, results in statistically significant reductions in fucosylated protein (Chen, Keusch et al. 2012). As part of the same study it was found that eliminating one or more tryptophan residues closest to the AB loop also results in significantly decreased fucosylation of the TSR, theoretically eliminating the $n$-related displacement of electrons which helps stably position the TSR in the central cavity of POFUT2(Chen, Keusch et al. 2012). Given the structural similarity of ADAMTSL2 TSRs to previously studied TSRs (in this case TSR2 & 3 of human Thrombospondin-1, and TSR2 of ADAMTS13) it is reasonable to predict that mutations affecting AB loop structure or the recognition site of POFUT2 will effectively reduce secretion of ADAMTSL2 as well. The ADAMTSL2 TSR6 mutants assayed in this study all exhibited defects in protein secretion. We had predicted that secretion defects would be the result of inadequate recognition by POFUT2. However given that both mutants which are predicted to disrupt $N$-glycosylation caused significant reduction in cellular and secreted protein, it is more likely that the $N$-glycosylation by OST is taking place. This is logical due to the face that $N$-glycosylation takes place in the lumen of the ER co-translationally, prior to peptide exposure to POFUT2(Dempski and Imperiali 2002). GD-like mutations assayed in this study occur at positions predicted to tolerate variability. However, the decrease in secretion observed (Figure 5) shows that there is a limit to this tolerance. This limited tolerance of variability at these positions may be caused by disruption in the structure of the AB loop. $O$-fucose site mapping as well as cell based assays using a bioorthogonal fucose analog, method described in Al-Shareffi, et al, showed a loss of fucosylation resulting from the S641L mutation in TSR3, (Melief C, unpublished observations)(Al-Shareffi E 2012). Previous studies using fucose deprived
Lec13 cells and siRNA knockdown of POFUT2 have shown that fucosylation of thrombospondin type 1 repeats was necessary for efficient secretion of ADAMTSL1 and ADAMTS13 (Ricketts LM 2007; Wang 2007; Luther KB 2009). Along with this evidence, showing the S641L mutation eliminates secretion and fucosylation, we can definitively state that $O$-fucosylation of TSR3 is necessary for protein secretion. And it is very likely that this defect in fucosylation is a cause of GD via dysregulation of TGFβ signaling in the ECM.

**Future Directions**

These experiments show that $O$-fucosylation sequon is important for efficient protein secretion. Currently, mass spectral analysis of all mutant clones is underway to determine whether or not ADAMTSL2 carrying mutations to $O$-fucose site can be fucosylated by POFUT2. A recent study utilized a novel method of liquid chromatography and mass spectroscopy to determine efficiency of fucosylation in the context of various mutations to TSR4 of F-SPONDIN relative to wild-type (Chen, Keusch et al. 2012). Experiments using this method can potentially allow us to determine the efficiency of fucosylation as a result of our previously discussed mutations to the $O$-fucose site. I expect that this type of assay would reveal a useful correlation between the percent of fucosylated product and the percent of total protein secreted, validating the functional importance of fucosylation as well as explaining the variation in efficiency of secretion.

We have shown that a small percentage of mutant ADAMTSL2 is secreted to the ECM. This observation may be eschewed by the nature of cell culture work, what we
interpret as secreted protein may have come from a small proportion of cells which have lysed in culture prior to collection of media. In order to complement the secretion assay presented in this thesis it may be logical to consider immunostaining transfected cells to qualitatively assess levels of retained vs secreted tagged ADAMTSL2 protein. This experiment may also help address whether or not mutant ADAMTSL2 is degraded. Judging by the lack of accumulated intracellular protein observed in this study, it is reasonable to predict that mutant ADAMTSL2 is degraded at an appreciable rate and should either be visualized in lysosomes or colocalized with proteasome complexes. In this way we can provide more evidence validating the importance of O-fucosylation in secretion of ADAMTSL2.

Though the addition of carbohydrates is important for efficient processing and secretion; does this particular modification have a role in protein-protein interaction in ECM? Studies showing the importance of ADAMTSL2 in TGFβ signaling were only published very recently. It is not yet known to what role if any carbohydrate modifications, namely those on TSRs, may play in binding LTBP1 and FBN1. Does the aberrant TGFβ signaling exhibited in GD patients’ arise from lack of secreted ADAMTSL2 or does it arise from the inability of unfucosylated to functionally regulate signaling? In order to carry this out one would need to generate a large amount of purified mutant ADAMTSL2 and perform coimmunoprecipitation and surface plasma resonance with its previously mentioned binding partners, LTBP1 and FBN1. Another remaining question concerns the endogenous levels of protein and their secretion. Since our assays make use of overexpressed protein in cell culture it will be helpful to observe the same effects in vivo. Presently I have not found any literature which
attempts to quantitatively characterize endogenous levels of ADAMTSL2. Since GD is not caused by haploinsufficient levels of ADAMTSL2 it is not likely that high levels of protein are needed to achieve proper TGFβ signaling regulation.

This study clearly demonstrates the role for the O-fucosylation consensus site for ADAMTSL2 secretion. TSR proteins require O-fucosylation for efficient processing and secretion by POFUT2 (Ricketts LM 2007; Wang, Dlugosz et al. 2007). Studies of POFUT2 deficient mice further validate the importance of O-fucose modifications to TSRs (Du, Takeuchi et al. 2010). We provide evidence to both validate and expand on previous studies’ showing the functional link between GD mutations in ADAMTSL2, protein secretion, and a resulting disruption of signaling ultimately giving rise to the disease phenotype (Le Goff, Morice-Picard et al. 2008). Using our novel TSR6 mutants; N813Q, T815V, and N813Q/T815V we can determine whether N-glycosylation or O-fucosylation is occurring on TSR6 and which process is more vital for efficient secretion. Our results show that all mutations cause defective secretion to varying degrees as well as variation in retention of protein. We show that substitutions primarily affecting the N-glycosylation sequon cause a more severe decrease in protein levels in both media and cell lysates, indicating rapid degradation. This data has implications on the relationship between different types of sugar modifications on TSRs, but further experiments on these special type of post translational modification will be invaluable to our understanding of protein processing and the functional link to diseases such as GD.
Materials and Methods

Site Directed Mutagenesis

*Wild-Type* myc 6x His tagged PCDNAmTSL2 previously described in (Koo BH 2007) and cloned into pSecTag hygroC vector. This plasmid was then used as a template for PCR based site directed mutagenesis. PCR was performed using 2.5U of Herculase II high fidelity DNA polymerase (Agilent Cat# 600675) to amplify 40ng of pSecTag-AdamsTs12 plasmid in a 20μl reaction volume. Since the plasmid has approximately 58% GC content, dimethyl sulfoxide (Baker Cat No# 9224-01) was added to the PCR reaction for a final concentration of 0.25%. Cycling conditions were set for a 95°C melting temperature for 30 seconds, 60°C annealing for 1 minute, 72°C extension for 8 minutes, and was repeated for 18 cycles. The PCR reaction was then treated with 10U of *DpnI* I restriction enzyme for 8 hrs to remove parental plasmid. A third of total reaction volume was then used to transform 50 μl of DH5α competent cells (apprx 3600 c.f.u.), cultured on LB + Agar (2.4%) plates with 100μg/ml Ampicillin (Sigma Cat #A9518-5G) at 37 °C overnight. Plasmid DNA was extracted from single colonies using Qiagen® QiaPrep Spin Mini Prep Kit (Qiagen Cat # 27106), and sequenced using primers listed in Table 2 to confirm mutation. Colonies with intended mutations were then regrown for larger scale plasmid DNA extraction using Qiagen® HiSpeed Plasmid Maxi Kit (Qiagen Cat # 12662). At this point, the entire mAdamsTs12 insert and flanking vector DNA were sequenced using primers listed in Table 2.

Secretion Assay

Cell Culture
293T cells were cultured in 10 mls Dulbecco’s Modified Eagle Medium (DMEM) (Gibco Cat#11965-092) supplemented with 10% bovine calf serum (HyClone) and 1% penicillin streptomycin antibiotic (Gibco Cat#15140-015), in 100 x 15mm sterile cell culture dishes grown in humid conditions at 37°C and 5% CO₂. Each dish of 293T cells (90-95% confluence) were split using 1 ml of 0.05% trypsin (Gibco Cat #15400-054), resuspended in 10mls of DMEM and subsequently added to X sterile 100 x 15mm dishes containing 9mls of DMEM every 2-3days to prevent overgrowth.

Transfection

293T cells grown to approximately 100% confluence were incubated in 1ml of .05% trypsin for 3 minutes, after which 9 mls of DMEM was added. Cells were then pelleted at 1500rpm for 5 minutes, resuspended in 12ml of DMEM and seeded in # of plates #6 well cell culture plates, and cultured for 24h prior to transfection. Transfection was carried out as follows; 0.8µg mAdamtsl2 and 0.2µg of hIgG was added to 100ul of Opti-MEM (Gibco Cat#31985) reduced serum media with 6 µl of polyethylenimine (PEI) (Sigma Cat#408727) and incubated at room temperature for 20min separate from 293T cells. During incubation, cell culture media (DMEM) was aspirated from the plated cells and replaced with Opti-MEM. DNA and PEI in Opti-MEM was added drop-wise to the cells and incubated for four hours at 37°C before aspirating and replacing media with fresh Opti-MEM. Cells were cultured for exactly 48hrs after transfection until collection of total 1 ml of media and 1 ml lysate was performed. Cells were lysed using standard chilled Radioimmunoprecipitation assay (RIPA) lysis buffer with added complete protease inhibitor cocktail tablets (1 tablet/10mls of buffer)(Roche Cat#04693116001) at 4°C for 20min. Collected media and cell lysates were stored in X ml aliquats at -20°C.
until protein content was determined using western blot. Protein was then visualized
and quantitated by Western Blot.

Western Blot

Lysate samples were acetone precipitated to 3x concentration using chilled (-
20°C ) acetone and incubated at -20°C for 2.5hrs and resuspended in sample buffer
(4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and
0.125M Tris HCL pH 6.8). 10μl of 2x loading dye was added to each sample. Samples
were boiled at 105°C, sonicated, and centrifuged at 14,000rpm for 5min. Samples were
then used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
PAGE separation of protein was performed using 4% polyacrylamide (PA) stacking gel
at 60mV into 8% PA separating gels run for 1hr 45min at a constant 140 mV. Samples
were transferred from SDS-PA gel to 0.45 μm nitrocellulose membrane (VWR
Cat#28155-900) at a constant 400 mA for 1hr in transfer buffer (10% methanol, 25mM
Tris, 192mM glycine). Membranes were blocked overnight at 4°C in 5% dry non-fat milk
in phosphate buffered saline with 0.1% tween-20 (PBST). The membrane was then
incubated in 1:2000 α-myc antibody (9E10 Sigma Cat# M4439-100UL) and 1% dry milk
in PBST for 1 hr at 25°C, followed by 4 15 minute washes in PBST at 25°C. This was
followed by a 1 hour incubation in 1:2000 Alexa Fluor 680 Goat α-mouse Ab (Invitrogen
Cat# A20186) and 1:5000 IRDye α-hlgG Ab (LI-COR Cat#926-32232) in PBST for 1
hour at 25°C, and subsequently followed by an addition 4 washes in PBST for 15
minutes each. Membrane fluorescence was imaged using Odyssey Infrared imaging
system from LI-COR Biosciences. Fluorescent intensity of hlgG and Alexa Fluor Ab
bands was quantified by Odyssey Imaging Software. Fold change was obtained by
taking raw values normalized to corresponding hlgG and divided by wild-type. All transfections were performed in triplicate to ensure consistency. Error bars indicate standard error of the mean.

Works Cited


Koo, B. H., et al. (2007). "ADAMTS-like 2 (ADAMTSL2) is a secreted glycoprotein that is widely expressed during mouse embryogenesis and is regulated during skeletal myogenesis." Matrix Biol 26: 431-441.


Figure 1. Clinical Manifestations of Geleophysic Dysplasia may result from impaired TGFβ regulation in the extracellular matrix. Figure 1 is adapted from Le Goff, et al. 2011; Allali & Le Goff et al., 2011; Bader et al., 2010; and Le Goff et al, 2012. A. X-rays of GD patients: (a) Posteroanterior hand X-ray at 3 and 10 years old. Note shortened tubular bones (asterisks). (b) Anterior Hip X-ray of 8 mo old, note widened epiphyses (arrowheads). (c) Lateral view vertebral column of 1 yr old GD patient, note ovoid vertebral bodies (OV). B. GD patients at 5 and 15 years of age exhibiting characteristic facial features and skeletal manifestations of the disorder. Note: (a) distended stomach (result of hepatomegaly), (a,b,c) upturned corners of the mouth, (d,e) broadened phalanges, and (f) epiphyseal dysplasia. C. Mutations in Fibrillin-1 (FBN1), ADAMTS2, and ADAMTS10 are proposed to alter TGFβ signaling and are part of a family of growth disorders. Increased TGFβ signaling (indicated in red) is proposed to lead to short stature, short hands, and stiff joints observed in Weil Marchesani Syndrome (WMS), Geleophysic Dysplasia (GD), Acromelic Dysplasia (AD), and Myrhe Syndrome (MS). Decreased TGFβ signaling (indicated in blue) is proposed to lead to development of tall stature and thin habitus observed in Marfan syndrome, Loeys-Diets Syndrome (LDS), and Camurati-Engelmann Disease (CED). Other abbreviations are as follows: latent TGFβ binding.
Figure 2. ADAMTSL2 TSRs was modified by POFUT2 at the consensus sequence CX\textsubscript{2}\textsubscript{3}(S/T)CX\textsubscript{2}G. ADAMTSL2 is a secreted glycoprotein with Thrombospondin Type I repeats (TSRs). TSRs often carry consensus sequences predicted to be glycosylated. A. Christina Leonhard-Melief used Sweet Software (REFERENCE) to model of sugar modifications on Thrombospondin 1 TSRs 2 & 3 (Tan, Duquette et al. 2002) (pubmed ID: 12391027). TSRs consist of approximately 60 amino acids with 6 conserved cysteine residues (grey) forming 3-disulfide bonds (yellow). In the ER, POFUT2 recognizes the loop structure (bracketed) containing the consensus CX\textsubscript{2}\textsubscript{3}(S/T)CX\textsubscript{2}G, and adds O-fucose (Red) to the central serine or threonine (turquoise). In the golgi, \(\beta\)-1,3-glycosyltransferase (\(\beta\)3Glc-T) further elongates the fucose by addition of a glucose (blue) to generate a disacharide. B. Schematic representation of the ADMATLS2 domain structure showing N terminal signal peptide, Cysteine Rich module, N glycan-rich module, PLAC domain, and 7-thrombospondin type I repeats (TSR - ovals). Six out of the seven TSRs have a predicted site of O-fucosylation (Green) and one has a predicted N-glycosylation site as well an O-fucose site (Yellow). Only one TSR lacks either consensus sequence (white).
Figure 3. Sequence confirmation of mouse *Adams12* expression vectors with intended mutations to TSR3 and TSR6. Mouse *Adams12* with either (A) Geleophysic Dysplasia-like mutations in TSR3 (S641L) and TSR6 (G817R) or (B) mutations to predicted O-Fucose and N-Glycan modification in TSR6: N813Q, T815V, and N813Q/T815V were cloned into pSecTag expression vector and sequenced completely to verify substitutions and integrity of clones. Mutated TSRs are listed to the left. Nucleotide substitutions and expected codon changes (start methionine defines nucleotide position 1) are indicated in the middle. Comparison of wild-type and mutant DNA sequence chromatograms for codons encompassing consensus sequences for O-fucosylation and N-glycosylation are shown on the right. Consensus sequence for O-fucosylation and N-glycosylation and translation of codons are indicated above chromatograms. Substitutions were made to Red amino acids. Threonine residues that are predicted to be modified by POFUT2 or oligosaccharyltransferase (OST) are indicated with an asterisk. Sequence data was analyzed using Sequencher 5.0 (GeneCodes™). Red brackets identify altered codons. All sequencing was carried out by Stony Brook University Office of Scientific Affairs DNA sequencing center.
Figure 4. Mutations to O-fucosylation/N-glycosylation consensus sequence in TSR6 disrupted protein secretion. A. Predicted sites of N-glycosylation and O-fucosylation and surrounding amino acids in Adamtsl2 TSR6 are conserved among vertebrate species. Consensus sequences for O-fucosylation, CX₂₋₃(S/T)CX₂G, and for N-Glycosylation, NX(S/T), are displayed below the multiple alignment and are highlighted in yellow and green, respectively. The underlined threonine is predicted to be glycosylated. Triangles indicate positions of N813Q and T815V substitutions introduced into ADAMTSL2. B. Western analysis of ADAMTSL2 (red) expression compared to human IgG (green) controls. ADAMTSL2 mutations predicted to disrupt N-glycosylation (N813Q), both O-fucosylation as well as N-glycosylation (T815V), and the double mutant (N813Q;T815V) reduce secretion relative to wild-type (WT). Despite decreased secretion levels, there is no significant increase in intracellular ADAMTSL2. C. Quantitation of ADAMTSL2 relative to hIgG controls in 293T culture media and cell lysate. ADAMTSL2 levels were normalized to hIgG and are plotted as a fraction of WT. All transfections performed in triplicate, Error bars indicate standard error of the mean (s.e.m.), and p values are indicated above each column.
Figure 5. Geleophysic Dysplasia-like substitutions in mouse ADAMTSL2 impaired secretion. A. Multiple alignment of TSR3 and TSR6 identifies conservation of predicted O-fucosylation sites and surrounding residues among vertebrates. Consensus sequences for O-fucosylation, $\text{CX}_{2-3}(\text{S/T})\text{CX}_2\text{G}$, and for N-Glycosylation, $\text{NX}(\text{S/T})$, are displayed above the multiple alignment and are highlighted in yellow and green, respectively. The underlined threonine is predicted to be glycosylated. B. Western Blot analysis of wild type (WT), TSR3 mutant S641L, and TSR6 mutant G817R (red) as well as hlgG secretion/transfection controls (green). GD-like mutations reduced in protein secretion. In contrast, intracellular levels of WT and mutant ADAMTSL2 are present at similar levels. C. Quantitation of ADAMTSL2 normalized to hlgG controls in 293T culture media and cell lysate, demonstrates that both GD-like mutations significantly reduce protein secretion. All transfections performed in triplicate, Error bars indicate s.e.m., and p values are indicated above each column.
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Table 1. Primers used for Site Directed Mutagenesis

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**Appendix B.** ADAMTSL2 TSR6 mutant secretion data taken 7/25/2012