ESTABLISHING GENOTYPE-PHENOTYPE RELATIONSHIPS IN POMGNT1 ASSOCIATED WITH CONGENITAL MUSCULAR DYSTROPHY

by

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(Under the Direction of Lance wells)

ABSTRACT

Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1(POMGnT1) is an influential glycosyltransferase required for modifying α-dystroglycan. Mutations in POMGnT1, removing GlcNAc modifications, have been associated with muscle-eye-brain (MEB) disease, a congenital muscular dystrophy. There are a number of varying phenotypes associated with MEB disease, corresponding to different point mutations in POMGnT1. In this study specific point mutations of POMGnT1 were selected to be examined, such that phenotype-genotype relationship can begin to be established. Using mass spectrometry and radiolabeled UDP-GlcNAc we were able to confirm that rPOMGnT1 was catalytically active. Through our analysis, we were able to compare the maximum initial velocity of the mutants as well as their thermal stability. Additionally, through homology modeling, predicted changes in tertiary structures could be associated with a loss of function. This data suggest that there could be a correlation between POMGnT1 expression and efficiency and disease phenotype.

INDEX WORDS: POMGnT1, post-translational modifications, α-DG, congenital muscular dystrophy

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B.S., Mercer University, 2009

B.A., Mercer University, 2009

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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August 2013

DEDICATION

To My Wife.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

THE DISEASE –

Muscle-eye-brain (MEB) disease, an autosomal recessive disorder, is a rare, but typically severe, form of congenital muscular dystrophy. Individuals with this condition are born with muscle weakness (hypotonia), ocular irregularities, and brain abnormalities [1-4]. Although worldwide epidemiological data for this myopathy is not available, regional estimates suggest that as many as 1 in 150,000 individuals are afflicted with MEB [5]. Reduced muscle tone and control is usually identified at birth by "floppy baby syndrome", characterized by the inability to properly control their heads and maintain flexed ligaments [6]. Effected individuals that survive past infancy display varying degrees of hypotonia from decreased fine motor control to severe muscle wasting. Some individual display pseudohypertrophy, where a loss in muscle mass is replaced with fatty or fibrous tissue, a phenotype commonly but not exclusively observed in patients with Duchenne or Becker's muscular dystrophy [7]. MEB patients may also exhibit ocular abnormalities including progressive myopia, retinal degeneration, and congenital glaucoma, ultimately leading to visual failure [2]. A hallmark of the disease is a developmental delay or intellectual disability, accompanying a change in brain morphology such as hydrocephalus and cobblestone lissencephaly [4]. Although there are phenotypes specific to MEB disease, there is a large overlap in characteristics of muscular dystrophies as a whole.

DYSTROGLYCANOPATHIES–

Muscular dystrophy is an assorted collection of recessive genetic illnesses defined by the consecutive onset of weakness and wasting of skeletal muscle [8]. Mutations in a number of muscle related proteins have been associated with a wide variety of muscular dystrophy phenotypes [9]. Duchenne muscular dystrophy (DMD), for example, is caused by a mutation in the gene encoding for dystrophin, which weakens the link between the ECM and cytoskeleton [10]. The absence of dystrophin from the DGC results in loss of stability of the sarcolemma making it more vulnerable to injury during muscle contraction. Muscular dystrophies can be further subdivided into more specific subtypes particular to affected proteins; collagen, laminin and dystroglycan [11]. A subset of these related disorders are the congenital muscular dystrophies (CMD) in which individuals are stricken with muscular dystrophy symptoms from birth due to disruption of the dystrophin-glycoprotein complex (DGC) [9, 12]. These so-called dystroglycanopathies result from genetic mutations in confirmed or putative glycosyltransferases involved in the O-mannosylation pathway and result in hypoglycosylated a-dystroglycan that is deficient in its ability to bind extracellular matrix components [12]. Interestingly, of the more than 40 different types of muscular dystrophy, it is predominantly only the dystrophies associated with O-mannose glycosylation defects that have significant neurological phenotypes [13]. Although this could be in part due to improper glycosylation of α-dystroglycan in the developing brain, the complete knock-down of α-dystroglycan in the neural tissues of mice fails to fully recapitulate the same phenotype as mouse models that are incapable of O-mannosylation [14].

DYSTROPHIN-GLYCOPROTEIN COMPLEX –

The Dystrophin-Glycoprotein Complex (DGC) is a large, multicomponent complex that is involved in both cell signaling and mechanically stabilizing interactions between the extracellular matrix (ECM) and the cytoskeleton of muscle [15-17] (Figure 1-1). Skeletal muscle, in particular, characteristically undergoes a significant degree of mechanical strain and cellular deformation with every contraction. Therefore, to preserve normal skeletal muscle function in an individual, this tissue must be able to routinely undergo cellular stresses required for movement while limiting mechanical cellular injury and adapting to changing workloads [18-20]. This is mediated by the DGC during normal muscle contraction; during a signaling event, when the sarcomere is contracted, the contractile force is transferred from actin to the fibrous ECM through protein intermediates. The is composed of numerous membrane and peripheral proteins, and the chief components include the dystroglycan complex, sarcoglycans, syntrophins, dystrobrevin complex, laminin and dystrophin [21]. Mutated, under expressed, and hypoglycosylated members of this complex are linked to increased cancer metastasis, arenavirus entry, and muscular dystrophy [22-24]. Although mutations in all of the above proteins can be associated with muscular dystrophies, the dystroglycanopathies are characterized by the hypoglycosylation of α-dystroglycan, necessary for its normal function as an extracellular-matrix protein.

Dystroglycan is the cornerstone of the dystrophin-glycoprotein complex, acting as an indirect link between the extracellular matrix and the internal actin cytoskeleton [15]. Dystroglycan is a central component of the DGC, and is produced by a single gene (DAG1), which is post-translationally cleaved into two subunits, a-dystroglycan and b-

dystroglycan [12]. Upon cleavage, the two subunits remain non-covalently associated and participate as components of the DGC complex to link the cytoskeleton to extracellular components [15]. a-Dystroglycan interacts with a host of extracellular matrix proteins, such as laminin, agrin, perlecan, pikachurin, and neurexin in a glycosylation dependent manner (Figure 1-1) [16, 25-28]. Properly O-mannosylated a-dystroglycan also serves as the receptor for several arenaviruses to gain entry into the cell [29, 30]. The O-glycan structures that decorate the mucin region of a-dystroglycan are a mixture of both O-GalNAc-initiated glycans and O-mannose-initiated glycans [13, 31]. Multiple sites of attachment have recently been elucidated including at least one site that bears a phosphorylated O-mannose structure [13, 31, 32]. While a primary amino acid sequence for O-mannosylation attachment has been proposed based on in vitro work, site mapping studies on endogenous protein from rabbit and human a-dystroglycan have demonstrated a more complex and less straightforward mechanism for selecting the site of O-mannose attachment in vivo[13, 31, 33]. Of particular interest, mutations in genes encoding the enzymes/proteins involved in the synthesis of O-mannosyl glycan structures disrupt the function of the DGC and are causal for various forms of congenital muscular dystrophy [23], as well as being associated with cancer metastasis in the case of at least one processing enzyme [22].

O-MANNOSE MODIFCATION –

The first mammalian O-mannose linked glycans were isolated from an enriched mixture of rat brain proteoglycans over three decades ago [34]. Subsequently, there has been much interest and investigation in cataloging a library of O-mannosyl-initiated glycans produced in mammalian tissues and it has been suggested that 30% of all O-

linked glycans in the brain are O-mannose structures (Figure 1-3) [31, 35-37]. The known structures are, for the most part, variations on a common tetrasaccharide core of Siaα3Galβ4GlcNAcβ2ManαSer/Thr. Common modifications of this theme include asialo-, or asialo-agalacto-, or fucosylated forms of the core, as well as a number of branched structures. A number of branched *O*-mannose-initiated structures have also been found in the brain with a GlcNAc at the 6-position of the core mannose [35, 38]. A unique *O*-mannose initated structure essential for protein function, laminin, and arenavirus binding by α-dystroglycan, has been partially characterized, that is, GalNAcβ3GlcNAcβ4(6-P-X)Man [32]. At the 6-position of the initiating mannose there is a phosphodiester linkage to an uncharacterized moiety, which is modified by LARGE with repeating units of [–3-xylose–α1,3-glucuronic acid-β1–] [39]. This novel structure, which appears key for α-DG function, has yet to be fully characterized nor have clear functional consequences been assigned to the classic or branched structures. However, as previously mentioned, glycosyltransferases known to modify *O*-mannose-initiated structures are associated with dystroglycanopathy disease phenotypes.

GLYCOSYLTRANSFERASES –

To date, mutations in seven glycosyltransferase or glycosyltransferase-like genes have been reported to alter the functional glycosylation of a-dystroglycan, and are causative for various forms of CMD [12]. These genes include: Protein O-mannosyltransferase 1 and 2 (POMT1 and POMT2) [40], Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) [41], Fukutin, Fukutin- Related Protein (FKRP), LARGE, GTDC2, and ISPD. While each of these genes was originally associated with a particular form of CMD, recent evidence suggests that different

mutations in the same gene can generate phenotypes associated with different forms of CMD arguing for some mutations being hypomorphic, as opposed to null, alleles. This makes a strong case for future efforts directed at elucidating genotype/phenotype relationships for this set of disease- causing genes. It should also be noted that approximately half of the patients diagnosed with various forms of dystroglycanopathy have no apparent mutations in the genes listed above suggesting that other disease-related enzymes in the O-mannosylation pathway remain to be discovered.

Protein O-mannosyltransferase 1 (POMT1) and 2 (POMT2) encode transmembrane ER proteins that are widely expressed in all mammalian tissues and catalyze the addition of an O-mannosyl residue from dolichol-phosphate-mannose onto Serine/Threonine residues of proteins in the secretory pathway [40, 42]. The expression of both POMT1 and POMT2 are required for proper O-mannosyltransferase activity in mammalian cells [33, 43]. Mutations in the genes of POMT1 and POMT2 were first identified in patients presenting with WWS, which is a severe, recessive form of CMD characterized by ocular abnormalities and severe brain malformations and a life expectancy typically less than one year [44]. However, mutations in these genes can also result in milder phenotypes of CMD such as muscle-eye-brain disease (MEB) [45].

Mutations in the fukutin gene are causative for Fukuyama congenital muscular dystrophy (FCMD), which among the Japanese population is one of the most common autosomal recessive diseases [23]. FCMD is associated with brain malformations due to defective neuronal migration [46]. While the exact function of the Golgi-localized fukutin remains unclear, the putative enzyme has sequence similarities with other glycosyltransferases including the DXD motif common to glycosyltransferases[47].

Similar to fukutin, FKRP encodes a putative glycosyltransferase belonging to the fukutin protein family. Mutations in the FKRP gene are causative for both severe and mild phenotypes of CMD, MDC1C, and LGMD2I, respectively [48]. In skeletal muscle, FKRP is localized to the sarcolemma of muscle fibers in association with the DGC [49]. It should be noted that both fukutin and FKRP do not fit into any known CAZY family of glycosyltransferases and contain LicD domains that are usually associated with enzymes involved in phosphorylcholine metabolism [50].

The LARGE gene, aptly named due to its more than 660 kilobase size, encodes a type II transmembrane protein located to the Golgi apparatus [51]. Recent evidence suggests that LARGE could act as a bifunctional glycosyltransferase, producing repeating units of [–3-xylose–α1,3-glucuronic acid-β1–] which allows α-DG to bind laminin [39] . Mutations in LARGE result in a substantial decrease in the molecular weight of a-dystroglycan as measured by SDS-PAGE, the inability of α-dystroglycan to bind to extracellular ligands such as laminin and are causative for CMD in mice (myodystrophy-LARGEmyd) and humans (MDC1D) [52-54]. Although the transfer reactions catalyzed by LARGE remains unknown, reports have indicated that LARGE is involved in the synthesis of glycan structures that are both on O-mannose-initiated structures as well as other types of glycans [55, 56]. Of exceptional interest, overexpression of LARGE can rescue laminin binding not only in LARGE-deficient cell lines but also in cell lines with deficiencies in the other known O-mannose pathway enzymes except for POMT1/2 [54]. Recently, the necessary extension (that has yet to be characterized) of the novel, laminin/virus-binding phosphorylated O-mannosyl trisaccharide structure on a-dystroglycan was shown to be LARGE dependent [32].

Other putative or known glycosyltransferases including ISPD, GTDC2, and B3GALNT2 have been associated with disruption of the dystrophin-glycoprotein complex and congenital muscular dystrophies [57-59]. Recessive mutations in a novel and uncharacterized gene, an isoprenoid synthase domain containing (ISPD) protein, have been identified in 9 patients with severe muscular dystrophy phenotypes [60]. Similarly, morpholino-mediated knockdown of zebrafish *gtdc2* replicates similar features of Walker-Warbug syndrome, a severe congenital muscular dystrophy [59]. B3GALNT2 is a known glycosyltransferase, although its role in the glycosylation of α-DG has yet to be elucidated, that has been identified in a broad spectrum of muscular dystrophy phenotypes in both patients and zebrafish knockdown models [61].

Protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1), the primary focus of this thesis, is a type II transmembrane protein that resides in the Golgi apparatus [62]. Mutations in POMGnT1 are causal for MEB, although some patients present with a WWS-like phenotype [41, 63]. It is responsible for the elongation of O-mannose modified glycoproteins with a GlcNAc in a β-1,2 fashion, according to the following reaction: UDP-GlcNAc + Man-R → GlcNAcβ1-2Man-R + UDP. The 75 KD human POMGnT1 (hPOMGnT1) protein consists of a cytosolic, a transmembrane, and a GnT-1 catalytic domain (Figure 1-2) and is ubiquitously expressed through most tissue types [41, 64]. Within the GnT I domain, which is 45% homologous with GnT I over 159 amino acids [64], POMGnT1 has a putative, conserved DXD motif, which participates in the coordination of divalent metal ions when binding nucleotide sugars [65, 66]. More than 20 point mutations in the transmembrane, stem and catalytic domains of POMGnT1 have been identified in MEB patients [4, 41, 63, 67, 68]. Recently, by utilizing a haploid

mammalian model five additional dystroglycanopathy gene candidates have been identified (SGK196, TMEM5, PTAR1, ST3GAL4, and B3GALNT2), in addition to all known genes. However, a link between severity of disease phenotype and gene mutation has yet to be drawn.

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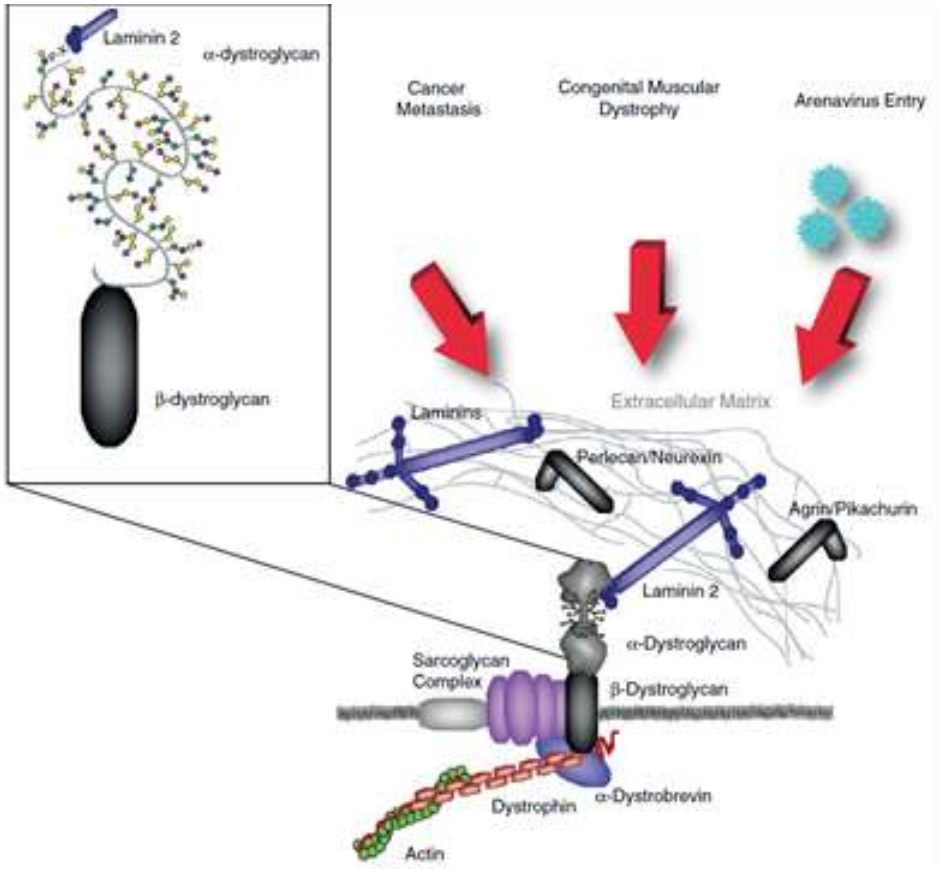

Figure 1-1. α –Dystroglycan and the dystrophin-glycoprotein complex (DGC).
α-Dystroglycan (α-DG), in a glycan-dependent manner, serves to bind extracellular matrix proteins as part of the complex and defects in O-mannosylation of this protein are associated with cancer metastasis, congenital muscular dystrophy, and arenavirus entry.

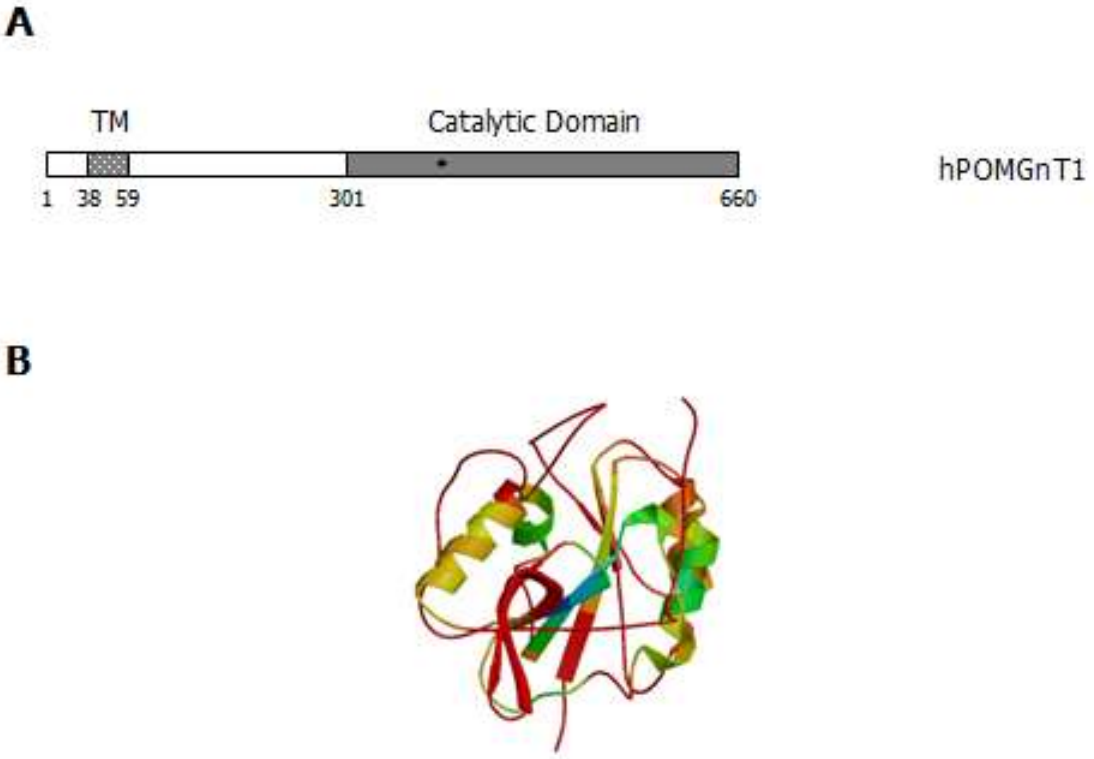

Figure 1-2. Schematic representation of human POMGnT1.
(A) Human POMGnT1 (hPOMGnT1) is a 75 kDa, typical type II transmembrane protein. In respect to the N-terminus it is composed of the following : a cytoplasmic tail, transmembrane domain (TM), stem domain, and catalytic domain. The catalytic domain contains a DXD motif (*), necessary for the coordination of divalent metal ions when binding nucleotide sugars. (B) Homology modeling of POMGnT1 based on the known crystal structure of GnT1 using SWISS-MODEL.

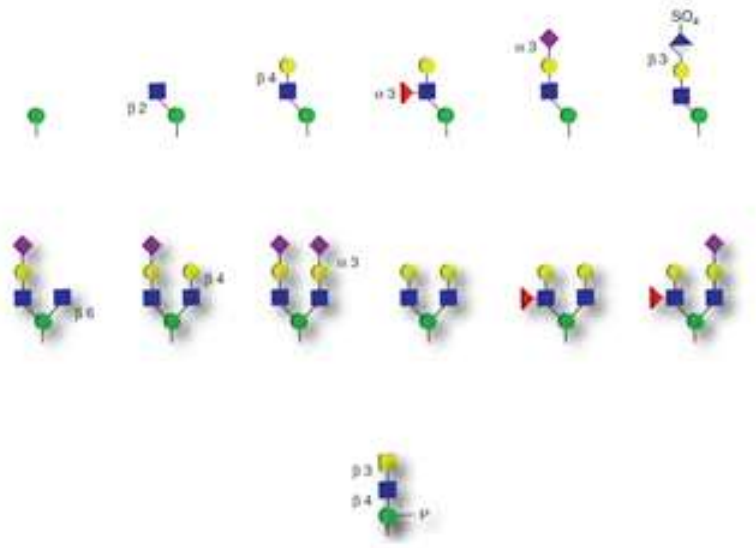

Figure 1-3. Diversity of mammalian O-mannose –initiated structures.
O-Mannose structures that have been observed included linear and bisected structures as well as a recently discovered phosphorylated mannose structure. Mannose (green circle), GlcNAc (blue square), galactose (yellow circle), fucose (red triangle), sialic acid (purple diamond), glucuronic acid (blue/white diamond), GalNAc (yellow square).

Table 1-1 Glycosyltransferases associated with dystroglycanopathies

Gene	Protein	Disease
POMT1	Protein-O-Mannosyltransferase 1	WWS, LGMD2K
POMT2	Protein-O-Mannosyltransferase 2	WWS, LGMD2N
POMGnT1	O-linked mannose β1,2-N-acetylglucosaminyltransferase	MEB, LGMD
LARGE	Large	WWS, MDC1D
FKTN	Fukutin	WWS, MEB-Like, FCMD, LGMD2M
FKRP	Fukutin-related protein	WWS, MEB-Like, FCMD, LGMD2I
ISPD	ISPD (isoprenoid synthase domain-containing)	WWS
GTDC2	GTDC2 (glycosyltransferase-like domain-containing 2)	WWS

WWS Walker-Warbug syndrome, *LGMD* limb girdle muscular dystrophy, *MEB* muscle eye brain disease, and *MD* muscular dystrophy

CHAPTER 2

GENERATION AND EXPRESSION OF RECOMBINANT HUMAN POMGnT1 PROTEIN ASSOCIATED WITH MEB DISEASE

INTRODUCTION-

Over the last three decades, since the initial description of patients with defects in protein glycosylation, over 40 types of congenital disorders of glycosylation (CDG) have been identified [1]. The advancement in this field coincided with the growing understanding of the significance and complexity of O-linked glycosylation [1-4]. Of particular note are the Congenital Muscular Dystrophies (CMDs), in which numerous genes encoding glycosyltransferases, both known and putative, have been shown to be causative for various disease phenotypes. These transferases have been associated with the addition and extension of O-linked mannose-initiated glycans [5, 6]. Mutations in one of these enzymes in particular, POMGnT1, are linked to a wide range of muscular dystrophy phenotypes.

Protein O-mannosyl β-1,2-N-acetylglucosaminyltransferase 1, or POMGnT1, has been shown to catalyze the second step involved in the synthesis of the sialyated *O*-mannosylated tetrasaccharide structure [7, 8]. This glycan structure, Siaα3Galβ4GlcNAcβ2ManαSer/Thr, has been identified on α-dystroglycan isolated from both brain and muscle. This glycan is not the laminin binding moiety and, in fact, removing the tetrasaccharide structure in a stepwise fashion with glycosidases enhances the ability of α-DG to bind laminin [9]. Almost two dozen recorded mutations in POMGnT1 have been associated with muscle-eye-brain (MEB) disease, a rare, recessive

dystroglycanopathy characterized by low muscle tone, ocular malfunction and mental retardation.

Given the importance of functional POMGnT1 in maintaining properly modified α-DG and the wide range of phenotypes observed in patients with MEB disease, we sought to identify and characterize the relationship between a genotypic mutation and the observed phenotype. Based on known mutations and disease phenotype, six point mutations were chosen that displayed a range of symptoms in patients (Table 2-1). However, before we could look at the enzyme kinetics we had to develop a POMGnT1 expression system to produce, and confirm, a highly pure recombinant POMGnT1 (rPOMGnT1) in mammalian cells.

EXPERIMENTAL PROCEDURES-

Site Specific Mutation of POMGnT1. The complete POMGnT1 gene used as a template for PCR amplification was (provided by Dr. Huaiyu Hu, State University of New York). The primer pair Fw (forward)/EcoRV (5′-GACACGAATTCATGTATCCATATGACGTCCC-3′) and Rv (reverse)/BglII (5′-CAGGAGCCCCAGAACAGACAAGATCTAGAACT -3′) was used in combination with Fermentas PCR 2X Master Mix to amplify the truncated sequence (amino acids 300-660) and introduce both flanked restriction sites and a N-terminal HA tag. Point mutations were introduced to the truncated POMGnT1 sequence using the QuickChange XL site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA) and cloned into the pFUSE-hIgG1-Fc2 secretory vector (Figure 2-1) (Invitrogen, USA). Finally, the constructs were both restriction digested and sequenced (John Hopkins Synthesis & Sequencing Facility) to confirm the introduction of the point mutations.

Protein Expression in HEK293 Cells and Purification. HEK293 cells were transfected with jetPRIME transfection reagent (Polyplus Transfection) using 5 µg of sample DNA per 10 cm cell plate. Cells were maintained in DMEM (Gibco) media supplemented with 10% FBS for 36 hours post transfection. Twenty-four hours prior to harvesting, cells were incubated in serum-free media.

To purify secreted POMGnT1 protein an immunoprecipitation was implemented. The cells were spun out of the media at 1000 RPM, and the media was filtered through a 0.2µ spin filter at 5000 RPM. A cocktail of protease inhibitors (1000X) was added and the total media volume was split up into 50 mL falcon tubes. Each sample was rocked for four hours at 4°C with 400 ul Santa Cruz Agarose A/G bead slurry, which has a binding capacity of 12 mg/mL and 25% bead volume. The samples were then combined by passing them over Micro Bio-Spin® Chromatography Columns (Rio Rad). The column was rinsed three times with PBS and protein was eluted twice with 900 µl of 100 mM glycine, pH 2.5, into 100 µl 1M Tris pH 8.5 for a total elution volume of 2 mL. Total protein was quantified by using Bio-Rad Bradford assay as previously described. The samples were then split, and 250 µl of 100% glycerol was added to each 1 mL aliquot. Purified POMGnT1 protein is stored on ice, in the 4°C walk-in refrigerator.

Silver Staining and Western Blotting of Gels. SDS-PAGE was performed on 7%, 4-15%, and 4-20% Tris-HCL precast gels purchased from Bio-Rad Laboratories. Silver staining was conducted using an adapted protocol from Shevenchko and colleagues [10]. Western blots were performed on PVDF membranes using anti-HA and FC antibodies followed by ECL detection.

RT-PCR. All primers were obtained from Thermoscientific and used with iQ SYBR Green Supermix (Bio-Rad). Amplifications were performed in a Bio-Rad 96-well iCycler or myIQ real time detection system using the appropriate iQ SYBR Green cycling protocol. Changes in target gene expression were normalized to the protein GAPDH. Relative transcript levels were calculated using the ΔΔCt method [11].

Protein Digestion. POMGnT1 purified from HEK293T cells was digested using sequence (Promega, USA) and diluted to 40 mM ammonium bicarbonate and reduced with 100 mM DTT for 1 hour at 56°C. Samples were then carboxyamidomethylated with 55 mM iodoacetamide in the absence of light for 45 minutes, and protease digested overnight at 37°C. Following digestion, the reaction was stopped using 1% trifluoroacetic acid. The resulting peptides were dired down using a Speed Cac and stored at -20°C until further analysis.

LC-MS and Data Analysis. Sample peptides were analyzed on a linear ion trap mass spectrometer (LTQ; ThermoFisher, USA). The peptides were resuspended in 19 µl of solvent A (0.1% formic acid) and 1 µl of solvent B (0.1% formic acid and 80% acetonitrile), filtered using a 0.2 µm spin filter at 12,000 rpm, and loaded on a C18 reverse phase column using a nitrogen pressure bomb. Peptides were eluted over a 160 minute linear gradient increasing from 5% to 100% B at a flow rate of 200 nl/min.

RESULTS-

POMGnT1 can be expressed and purified from HEK297T cells. To generate a truncated, thus secretable, and tagged recombinant POMGnT1 (rPOMGnT1), primers were used to clone rPOMGnT1 into a pFUSE secretory vector (Figure 2-2). Point mutations were made in the constructs, using site-directed mutagenesis that coincided

with a known point mutant previously described and associated with MEB patients (Table 2-1). These constructs were transiently transfected into HEK293 cells, and the secreted protein product was harvested from media. The relative rPOMGnT1 associated protein purified from the media was 2.5 mg at a current stock concentration of 1 µg/ µl. In all purified samples no bands were present on silver stains for two of the rPOMGnT1 constructs, R442C and C490Y, nor was there associated tag reactivity on the Western Blots.

rPOMGnT1 steady state levels do not change with variation in expression. Several mechanisms can account for the dissimilarity in expression levels of rPOMGnT1, including degradation or downregulation of the mRNA precursor. To test this, qPCR analysis was performed to address any difference in the steady state mRNA expression levels. Compared to wildtype rPOMGnT1 expression, alterations in transcript abundance did not appear to account for the lower protein levels in the R443C and C490Y mutants, as the level of POMGnT1 transcripts was comparable (Figure 2-3). This suggests that a substantial decrease in R442C and C490Y mRNA steady state levels is not associated with abolished protein secretion.

Sample purity was also confirmed by LC-MS/MS. To more conclusively determine the constituents present, the immunprecipitated samples were trypsin digested followed by LC-MS/MS (Figure 2-4). Based on the sequence of the rPOMGnT1, coverage at a 1% false-discovery rate (FDR) was between 20 and 67% depending on the mutant sample. POMGnT1 was identified in all six mutant samples, including the R443C and C490Y samples. Keratins and albumin were also identified in all of the samples but are only minor co-purifying/contaminating proteins. This characterization of mutant

POMGnT1 material affirmed that we could express rPOMGnT1, with two noted exceptions, and had relatively high sample purity.

DISCUSSION-

The importance of POMGnT1 and its role in properly glycosylating α-DG, and potentially other proteins, has been a focus of study since it was first correlated with congenital muscular dystrophies. Additionally, mutated, recombinant POMGnT1 has been truncated and expressed in a variety of ways. However, to better understand the effects known point mutation have on POMGnT1 structure and function a dependable expression system has been developed.

The POMGnT1 catalytic domain (GnT1-like domain) was cloned into a pFUSE secretory vector, flanked with HA and Fc tags (Figure 2-1, 2-2). This was advantageous for both harvesting rPOMGnT1 from cell grown media and protein purification by using the incorporated tags. Initial attempts at purifying recombinant POMGnT1 mutants by only anti-Fc immunoprecipitation yielded samples with low POMGnT1 purity and high contamination (Figure 2-3a). By western blot, all mutant samples, with the exception of R442C and C490Y exhibited reactivity for anti-HA antibody at the expected 75 kDa range (Figure 2-3b). To increase purity, the two-stage purification assay was implemented, and rPOMGnT1 sample purity increase six fold for the expressed mutants (Figure 2-3c). The mutants R442C and C490Y showed no improvement in relative protein expression or western blot reactivity. This is in contrast to a study suggesting that the C490Y mutant in a similarly truncated POMGnT1 construct is able to be expressed in e. coli [12].

To determine the cause the expression deficiency, mRNA expression levels were quantified and cytosolic samples were analyzed. Concurrently with harvesting media for

further protein purification, HEK293T cells were harvested and analyzed via qPCR (Figure 2-3). When compared to WT rPOMGnT1 there was a significant decrease in R442C steady state mRNA, however, this 10% decrease in transcript is unlikely causative for the complete abolition of expression. Following the pathway of protein production, cytosolic cell fractions collected for analysis to determine if mutant protein was not properly being secreted (Figure 2-4). Purified cytosolic protein from mutant rPOMGnT1 samples showed similar abundance of anti-HA reactive protein. These two pieces of evidence suggest that the absence of R442C and C490Y mutants' expression is not a function of either global mRNA steady state levels, or compartmentalization of protein in the cytoplasm.

For future catalytic and thermal analyses, purity of the mutant rPOMGnT1 samples is a high priority. Therefore, to determine the overall protein composition of the samples they were digested, analyzed by LC-MS/MS and proteins were identified by Proteome Discoverer (Figure 2-5; Supplemental Data). Overall, there was a high sequence coverage of rPOMGnT1, ranging from 20-67%(Figure 2-5 a; supplement)In all mutant samples, peptides corresponding to rPOMGnT1 were identified and, for two of the six mutants, the specific amino acid substitution could be confirmed (Figure 2-5b; Supplement). A list of all proteins identified were compiled and the two contaminating protein groups found to some degree in all samples were keratins and albumin (Table 2-2). Presumably, the keratin found in the samples is contamination from handling by the researcher introduced during the protein digestion and C-18 clean-up stages.

In conclusion, we have developed and implemented an expression and purification system for rPOMGnT1 in mammalian cells. The resulting protein samples

are of a relatively high purity, containing minimal protein contaminants, keratin and albumin. The mutant constructs that are expressed (A303V, R311G, D556N, and R605H) are reactive to anti-HA antibody via a western blot and have been confirmed by mass spectrometry analysis. Being able to produce rPOMGnT1 with point mutations coinciding with known muscle-eye-brain mutations, will allow for the further examination of the genotype-phenotype relationship of the disease.

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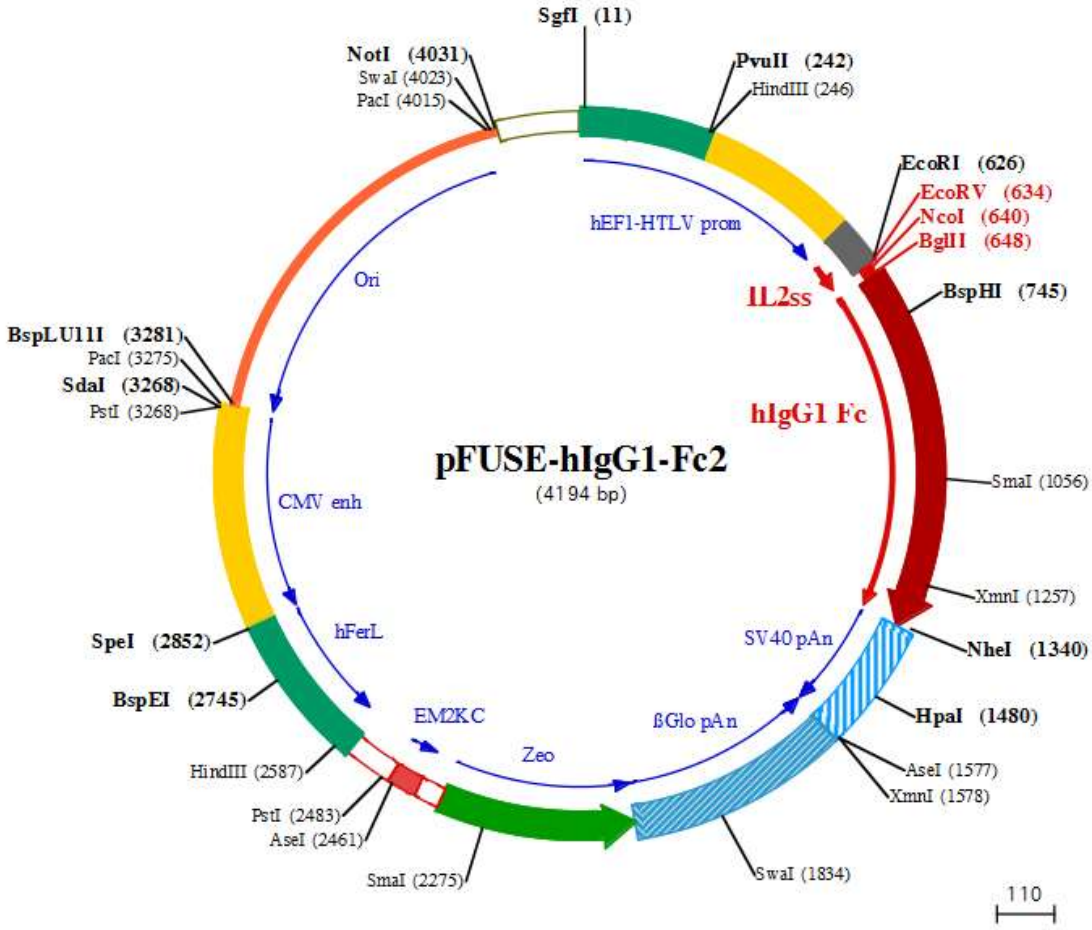


Figure 2-1. Schematic representation of human pFUSE vector IgG1-Fc2.
rPOMGnT1 expression vector that fuses a C-terminal Fc tag and a N-terminal IL2 secretion signal on all proteins. This facilitates secretion and purification of rPOMGnT1 from cell media using protein A/G affinity chromatography.

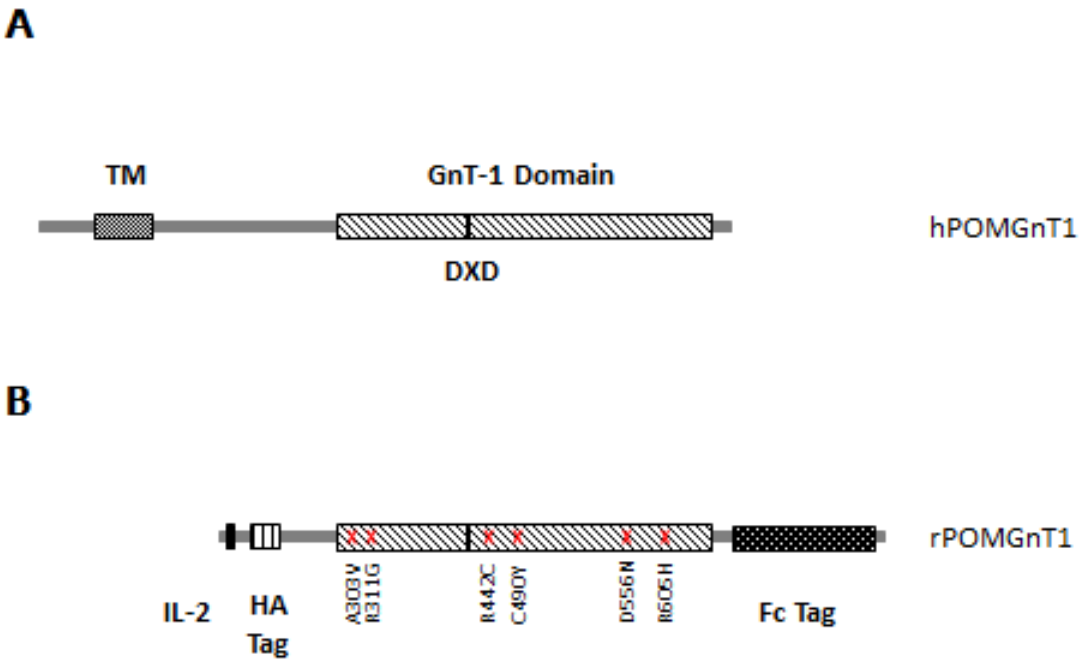
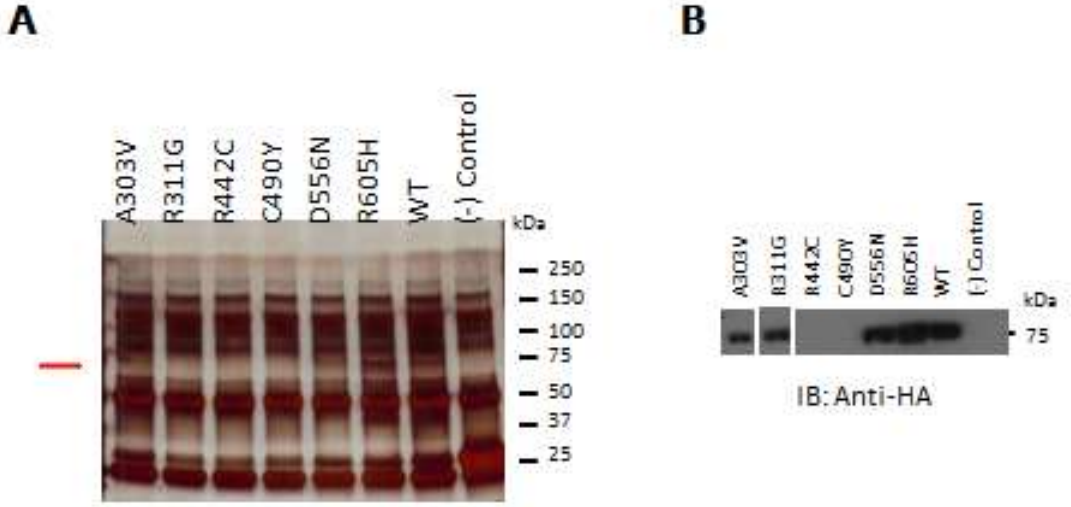
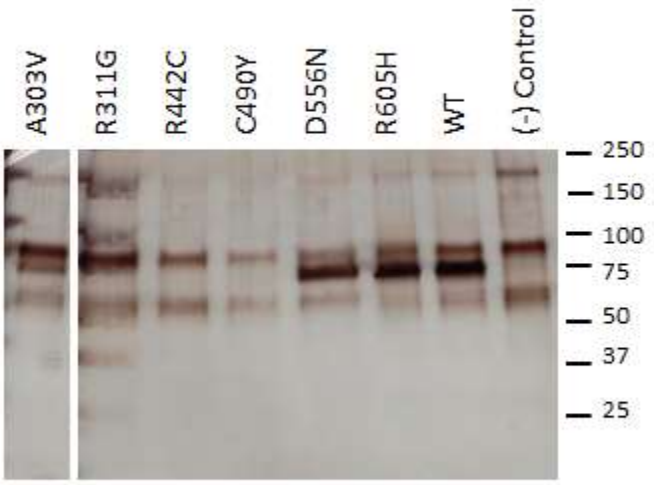


Figure 2-2. Native POMGnT1 in comparison with the recominant form.
(A) Schematic diagram of human POMGnT1 (hPOMGnT1). hPOMGnT1 contains an N-terminal transmembrane domain (TM), a stem domain, and a C-terminal GnT-1 domain, in which a conserved DXD motif is found. (B)Schematic of recombinant POMGnT1 (rPOMGnT1) with an N-terminal IL2 signal sequence and HA tag and a C-terminal Fc domain. The TM and stem domains have been truncated, and individual point mutations have been made (X), to develop a library of six individual mutants.


A
A303V
R311G
R442C
C490Y
D556N
R605H
WT
(-) Control
kDa
250
150
100
75
50
37
25
B
A303V
R311G
R442C
C490Y
D556N
R605H
WT
(-) Control
kDa
75
IB: Anti-HA

C


A303V
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WT
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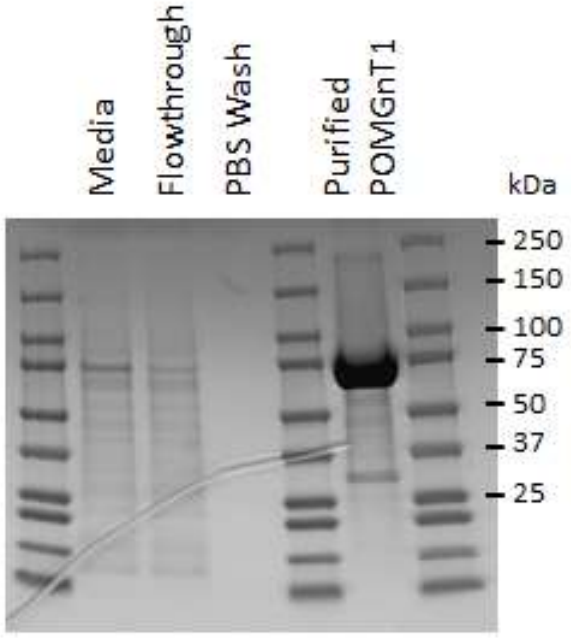
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Flowthrough
PBS Wash
Purified POMGnT1
kDa
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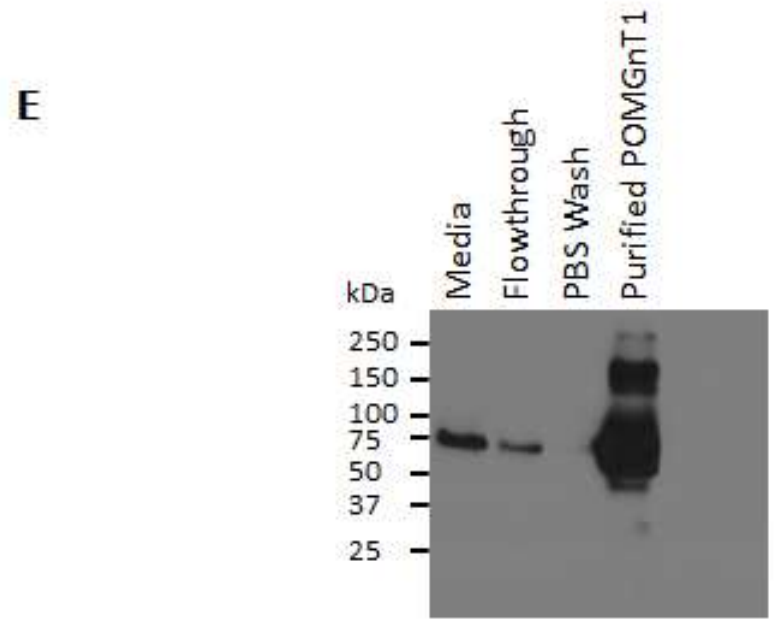

Figure 2-3. Purified recombinant POMGnT1 from HEK293T cells.
(A) Silver staining following SDS-PAGE of recombinant POMGnT1 (rPOMGnT1) after purification with protein A/G. Lanes 1-6 correspond rPOMGnT1 with respective mutations (A303V, R311G, R442C, C490Y. D556N, and R605H). The negative (-) control is sample purified from mock transfected cells. The 75 kDa band, corresponding to the secreted rPOMGnT1 is denoted by the red arrow. (B) Western blot analysis following SDS-page of purified rPOMGnT1 with the anti-HA antibody, specific for the incorporated HA tag. (C) Silver staining following SDS-PAGE of rPOMGnT1 after two-step purification with anti-HA antibody, followed by protein A/G.

A

B

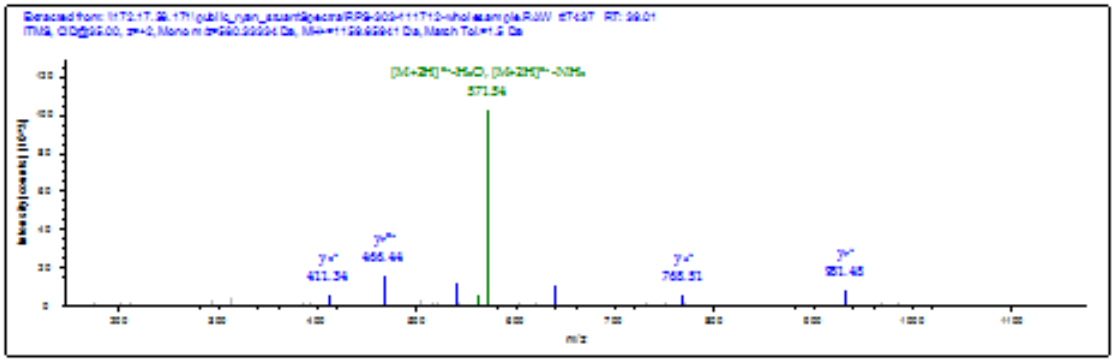

Figure 2-4. Mass spectrometry coverage of A303V mutant and confirmation of substitution.
Transcript abundance of rPOMGnT1 in HEK293T cells 3 days after transient transfection. Samples were normalized to GAPDH and compared to WT steady state levels.

Table 2-1

hPOMGnT1 Mutation	Phenotype	Clinical Aspects	References
D556N	Limb-Girdle Muscular Dystrophy	Very late onset (12 yrs), myopic, no ocular or brain abnormalities	Clement et al. *Arch Neurol (2008)*
R311G	Muscle-Eye-Brain Disease	Global hypotonia, cataracts, sever mental retardation	Biancheri et al. *Arch Neurol.* (2006)
R605H	Muscle-Eye-Brain Disease	Mild hypotonia, complete blindness, brain and neural abnormalities	Demir et al. *Neuro. Dis.* (2009)
A303V	Novel (MEB-like)	Hypotonia, glaucoma, brain abnormalities	Hehr et al. *Neurogenetics.* (2007)
R442C	Muscle-Eye-Brain Disease	Hypotonia, epilepsy, severe to profound cognitive and fine motor delays	Vervoort et al. *Ann Neurol.* (2004)
C490Y	MEB-Fukuyama CMD	Severe hypotonia, ocular abnomalities, early death	Godfrey et al. *Brain.* (2007)

Table 2-1. POMGnT1 point mutations and their associated diseases.
To evaluate a broad range of phenotypes within muscle-eye-brain disease, specific point mutants of POMGnT1 were chosen to be expressed for study. These mutants have known point mutations and phenotypes have been documented

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
RPS-Recombinant-POMGnT1-A303V	RPS-Recombinant-POMGnT1-A303V	245.57	62.95	1	36	36	69	672	76.8	6.35
229552	albumin [Bos primigenius taurus]	219.07	62.99	1	3	36	70	581	66.1	6.09
113574	SERUM ALBUMIN PRECURSOR.	216.18	57.17	1	7	36	70	607	69.2	6.11
136429	TRYPSIN PRECURSOR.	58.04	31.60	1	4	5	21	231	24.4	7.18
Trypa5	PromTArt5 Promega trypsin artifact 5 K to R mods (2239.1, 2914)(1987, 2003)	43.54	100.00	1	3	4	13	50	5.6	5.08
547749	KERATIN, TYPE I CYTOSKELETAL 10 (CYTOKERATIN 10) (K10) (CK 10)	40.91	19.39	2	13	13	13	593	59.5	5.21
1346343	KERATIN, TYPE II CYTOSKELETAL 1 (CYTOKERATIN 1) (K1) (CK 1) (67 KD CYTOKERATIN) (HAIR ALPHA PROTEIN) gi|186772 (M98776) keratin 1 [Homo sapiens]	35.82	14.91	1	8	10	14	644	66.0	8.12
254622	(S43646) cytokeratin 2, CK 2 [human, epidermis, Peptide, 645 aa] [Homo sapiens]	23.36	11.63	2	5	7	8	645	65.9	7.96
1082558	keratin 9, cytoskeletal - human gi|435476 (Z29074) cytokeratin 9 [Homo sapiens]	14.61	7.87	1	4	4	5	623	62.1	5.30

Table 2-2. Proteins identified in A303V purified recombinant POMGnT1 sample.

CHAPTER 3

CHARACTERIZATION OF POINT MUTATIONS ON RECOMBINANT POMGNT1 STRUCTURE AND FUNCTION

Stuart R, Stalnaker S, Wells L. To be submitted to The Journal of Biological Chemistry.

ABSTRACT –

Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1(POMGnT1) is an influential glycosyltransferase in modifying α-dystroglycan. Mutations in POMGnT1, removing GlcNAc modifications, have been associated with muscle-eye-brain (MEB) disease, a congenital muscular dystrophy. There are a number of varying phenotypes associated with MEB disease, corresponding to different point mutations in POMGnT1. In this study wild type recombinant POMGnT1 was selected to be examined, such that phenotype-genotype relationship can begin to be established. Using mass spectrometry and radiolabeled UDP-GlcNAc we were able to confirm that POMGnT1 was catalytically active. Through our analysis, we were able to compare the maximum initial velocity of the enzyme as well as the Km. Additionally, through homology modeling, predicted changes in tertiary structures could be associated with a loss of function. This data suggest that there could be a correlation between POMGnT1 expression and efficiency and disease phenotype.

INTRODUCTION –

Congenital muscular dystrophy (CMD) is a heterogeneous group of recessively inherited disorders characterized by muscle weakness, ocular and neuronal abnormalities, and mental retardation [1]. Within the last 20 years, it has become evident that the hypoglycosylation of α-dystroglycan (α-DG) is a common theme between the various diseases of CMD [2]. α-DG is the key component of the dystrophin-glycoprotein complex (DGC), and serves as a tie between the actin cytoskeleton of muscle cells and the extracellular matrix [3]. For α-DG to interact properly with its extracellular ligand, laminin, it must be properly post-translationally modified through the addition of O-

linked oligosaccharides [4, 5]. There are eight known or putative glycosyltransferase genes currently associated with varying forms of CMD in which the glycosylation of α-DG is affected [6]. The eight mutated genes are *protein O-mannosyltransferase 1* (POMT1), *protein O-mannosyltransferase 2* (POMT2), protein O-linked mannose β1,2 Nacetylglucosaminyltransferase (POMGnT1, Fukutin, Fukutin-related protein (FKRP), LARGE, GTDC2 and isoprenoid synthase domain containing (ISPD) protein [7-14]. Mutations in any of these genes can cause several forms of CMD, varying in phenotype, suggesting the phenotypic display is dependent on the severity of the mutation [15]. Muscle-eye-brain disease, is a prime example of this, as mutations in POMGnT1 have been identified as causal for all cases but there is a wide range of phenotypes displayed[16].

POMGnT1 is a type II transmembrane protein that resides in the Golgi apparatus, and is known to elongate O-mannose modified proteins with GlcNAc in a β-1,2 fashion [17]. This ubiquitously expressed protein has three main domains and, of which, the catalytic domain is homologous to that of GnT1 [8, 18]. Mutations in POMGnT1 are causal for MEB, although some patients present with a WWS-like phenotype [8, 19]. More than 20 point mutations in the transmembrane, stem and catalytic domains of POMGnT1 have been identified in MEB patients [8, 16, 19-21].

Given the variability of muscle-eye-brain pheynotype due to mutations in POMGnT1, we set out to draw a correlation between the genotype-phenotype relationships. To do this, we utilized purified recombinant POMGnT1 (rPOMGnT1) with known mutations specific for a range of MEB phenotypes. These mutant samples were analyzed for multiple factors including catalytic activity, maximum rate, and thermal

stability. Using these evaluation factors, we were able to identify these specific point mutations as expression, Vmax, and/or thermal stability mutants.

EXPERIMENTAL PROCEDURES –

Synthetic O-mannose Peptide

Synthetic peptide, (Ac-YVEPT[m]AV-NH2), was generously provided by Dr. David Live (University of Georgia, Athens GA).

Transferase Activity with Varying Reaction Time

The following protocol was performed for a 25 µl sample volume (Table 3-1): A master mix consisting of 5 µl water,2.5 µl 1 M Mes (pH 7.0) [100 mM Mes final], 2.5 µl of 100 mM $MnCl_2$ [10 mM $MnCl_2$ final], 2.5 µl 10% Triton X [1% Triton X final], 2.5µl of 1:50 UDP-[3H]-GlcNAc stock and 2.5 µl of 50 mM AMP [5 mM AMP final] was made as the reaction buffer for each sample. For each reaction, 17.5 µl of master mix was aliquoted. To each aliquot 2.5 µl of 200 mM UDP-GlcNAc [20 mM final], and 2.5 µl of 10 µg/µl synthetic o-mannose modified peptide (1 mM final) and 2.5 µg of purified POMGnT1 was added and immediately divided into 1.5 mL tubes to be analyzed for the following time points: 0, 30, 60, 90, and 120 minutes. Samples were vortexed before incubation. The samples were incubated for their respective times at 37°C and 100 µl of 1% TFA was added to stop the reaction, which was then stored on ice until analysis. Once all time points were completed, the samples were passed over a reverse phase C18 column. Sample was loaded 2x on the column, washed with 200 µl buffer A 3x and eluted with 200µl buffer B. The elutions were put directly into scintillation buffer and analyzed by a Beckman scintillation counter. This protocol can be found in the public domain of the WELLSLAB server, under protocols.

Transferse Activity with Varying POMGnT1 Enzyme Concentration

Similar to the time varyiation assay, a master mix consisting of 100 mM Mes (pH7.0), 10 mM $MnCl_2$, 1% Triton X, 5 mM AMP, UDP-GlcNAc (20 mM final), UDP-[3H]GlcNAc (20 µM final) and O-mannose peptide (1 mM final) was made. Of the master mix, 17.5 µl was aliquoted out for each reaction to which water (7.5, 6.5, 5.5, 4.5 and 3.5 µl) and enzyme (0, 1, 2, 3, and 4 µl respectively) was added. These samples were incubated at 37°C for 1 hour, quenched with 1% TFA, cleaned up over C18 column, and analyzed by scintillation counter as described above. This protocol can be found in the public domain of the WELLSLAB server, under protocols.

POMGnT1 UDP-GlcNAc Km Assay

A master mix consisting of 100 mM Mes (pH7.0), 10 mM $MnCl_2$, 1% Triton X, and 5 mM AMP was made as the reaction buffer. Additionally, O-mannose peptide (1 mM final) and UDP-[3H]GlcNAc (20 µM final) were added. The master mix was divided and varying concentrations of UDP-GlcNAc was added to each respective sample as follows: 0, 0.17, 0.25, 0.33, 0.5, 0.67, 1, 2, 4, and 8 mM UDP-GlcNAc. Water, to volume correct, and 2.5 µg of POMGnT1 were added to each sample and incubated for 1 hour at 37°C. The reactions were quenched using 1% TFA, cleaned up over a C18 column, and analyzed by scintillation counter all as previously described. This protocol can be found in the public domain of the WELLSLAB server, under protocols.

RESULTS –

Through both mass spectrometry based approaches and radiolabeling assays, we can conclude that the recombinant POMGnT1 retains the catalytic ability to add GlcNAc to an O-mannose initiated substrate (Figure 3-1, Figure 3-2). By mass spectrometry,

when reacted with substrates in the necessary reaction buffer, we were able to successfully identify the addition of a GlcNAc to O-man substrate by the recombinant POMGnT1 after 96 hours. We were able to track the catalytic ability of POMGnT1 by using UDP-[3H]GlcNAc and observe the labeling of the substrate by the enzyme over time (Figure 3-2). Additionally, by varying POMGnT1 enzyme concentration we were able to conclude that protein concentration was not having an effect on enzyme rate (Figure 3-3).

Finally, to determine POMGnT1 Km, UDP-GlcNAc substrate concentration was varied and tracked by radiolabeling assay. A substrate range of 0-8 mM was used, due to known Km values of WT and variations of recombinant POMGnT1 being 0.73 mM and 3 mM respectively. In analyzing our data via a Lineweaver-Burk plot, we were able to calculate that our recombinant POMGnT1 had a Km of 4.48 mM. The Vmax was calculated to be 4.9E10 mmol/min/ug POMGnT1.

DISCUSSION –

Point mutations in POMGnT1 have been linked through genetic analysis to a range of phenotypes of MEB disease. However, identification of the link between some of the genotypic mutations and the observed phenotype had yet to be elucidated. Therefore, we set out to generate and characterize recombinant POMGnT1 protein, and theorize how it can be directly correlated to disease severity.

Before we could analyze POMGnT1 we had to confirm that our recombinant POMGnT1 had the capacity to be catalytically functional (Figure 3-1, 3-2). A previous study has shown that all of these mutants can be expressed, in a slightly different recombinant form, in E. *coli* and some are catalytically active [18]. In this particular

study it was shown that the D556N mutant had similar activity to the wild type recombinant but that all other mutants analyzed showed no catalytic ability. Although in our study, when expressed in a mammalian cell line, not all mutant clones were expressed, we could confirm our POMGnT1 was catalytically active by mass spectrometry analysis (Figure 3-1). During a 96 hour radiolabeling assay, a majority of the expressed mutants were able to catalyze the addition of [^{3}H]GlcNAc to a synthetic substrate (Figure 3-2). This contradicts a previous study in which some of the same mutants were incubated for 16 hours and found to be catalytically dead [18]. The D556N mutant is the only mutant in both studies found to be catalytically active and, interestingly, is also the mutation in which the least severe phenotype is observed.

To quantify an effect on enzyme activity, the expressed POMGnT1 was analyzed for maximum initial velocity (Vmax) and Km. The samples were incubated with substrate peptide and varying amounts of UDP-GlcNAc and analyzed for reaction rate (Figure 3-4). Since the reported UDP-GlcNAc Km for POMGnT1 is 0.73 mM and other groups have reported similar values for recombinant POMGnT1, we used a comparable range of substrate concentrations for our analysis; 0-8 mM. Through analysis via a double reciprocal plot, we calculated a Km value of.0.48 mm.

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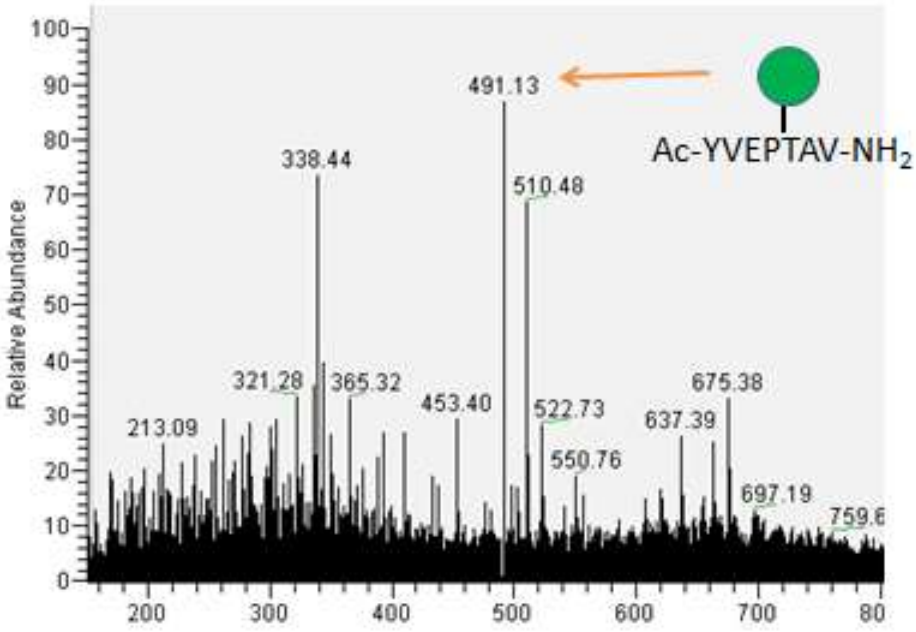
A


Relative Abundance
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90
80
70
60
50
40
30
20
10
0
200
300
400
500
600
700
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491.13
338.44
510.48
321.28
365.32
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522.73
675.38
637.39
213.09
550.76
697.19
759.6
Ac-YVEPTAV-NH2

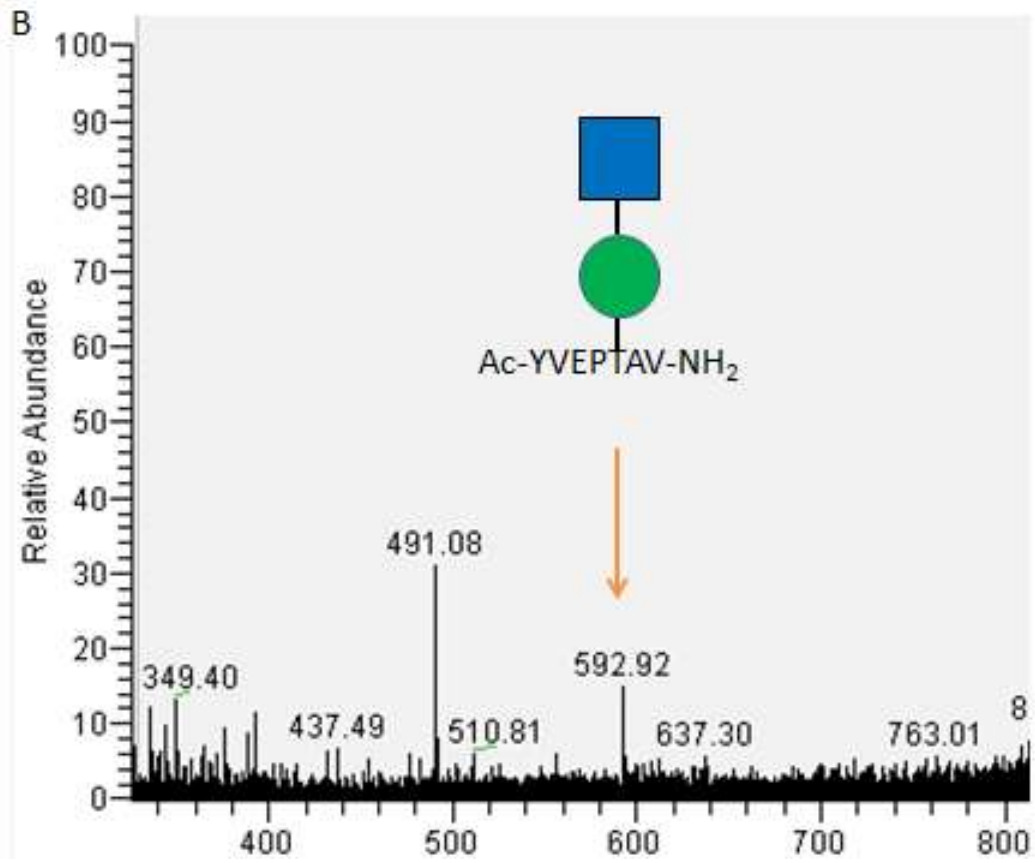


Figure 3-1. POMGnT1 mediated transfer of GlcNAc to O-mannose peptide.
Recombinant POMGnT1 (rPOMGnT1) was incubated with a known substrate, a synthetic O-mannose modified peptide for 96 hours under conditions to drive the reaction to completion. From the full length MS scan of unmodified peptide before introducing rPOMGnT1(A) we were able to identify the O-mannose peptide in the doubly charged state (m/z 491). Following the addition and incubation of rPOMGnT1, a peak corresponding to the doubly charged state corresponding to the addition of a GlcNAc was identified (B).

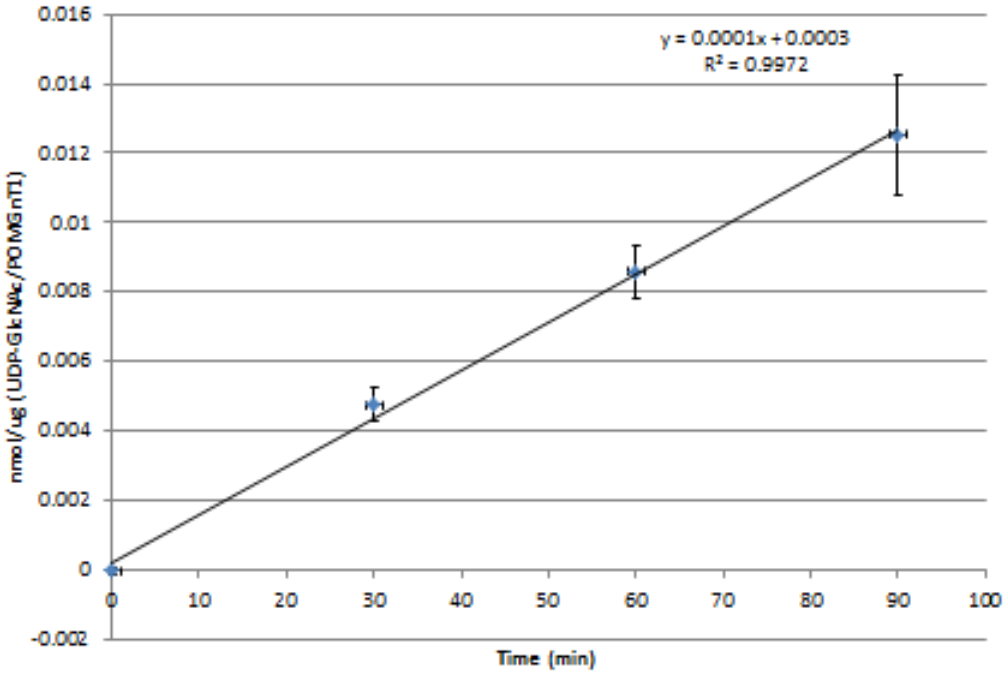


Figure 3-2. Effect of varying reaction time on POMGnT1 rate.
Recombinant POMGnT1 was incubated with known substrates, UDP-GlcNAc and an O-mannose modified peptide, for 4 hours in reaction buffer. Samples reacted for 0, 30, 60, 90 and 120 minutes respectively and the reaction was neutralized using 1% TFA. The peptide was cleaned up over a C18 reverse phase column, eluted and analyzed via scintillation counting. The reaction rate of UDP-GlcNAc addition per unit POMGnT1 continued to be linear throughout the 120 minute time point, as expected.

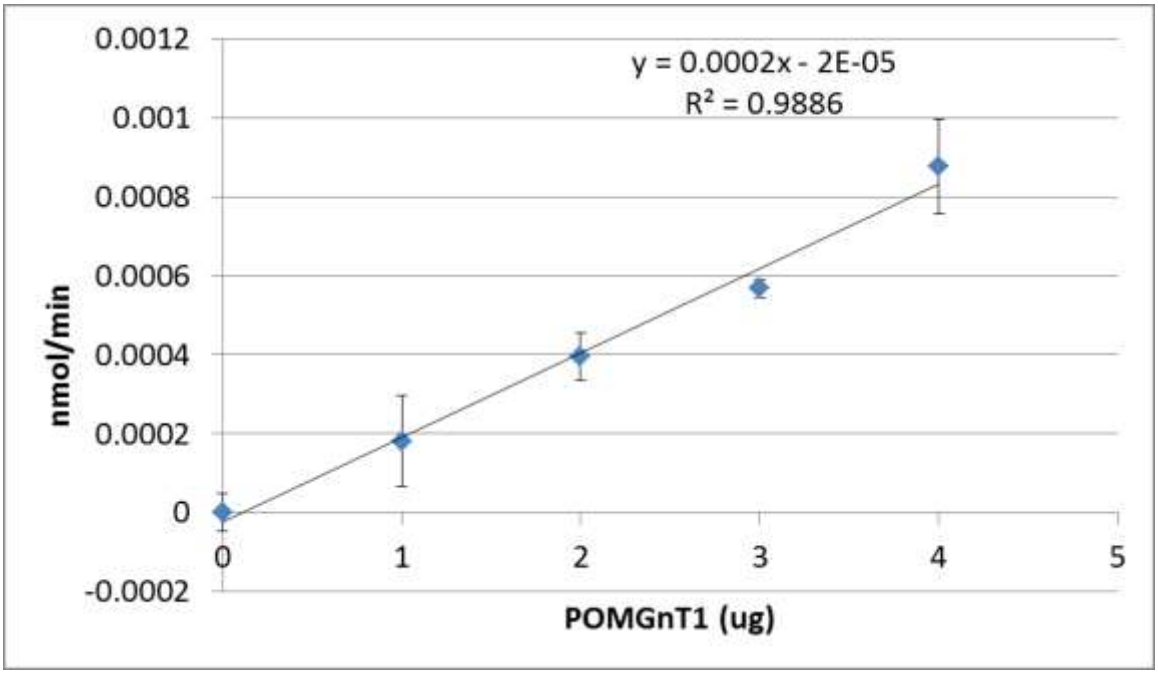
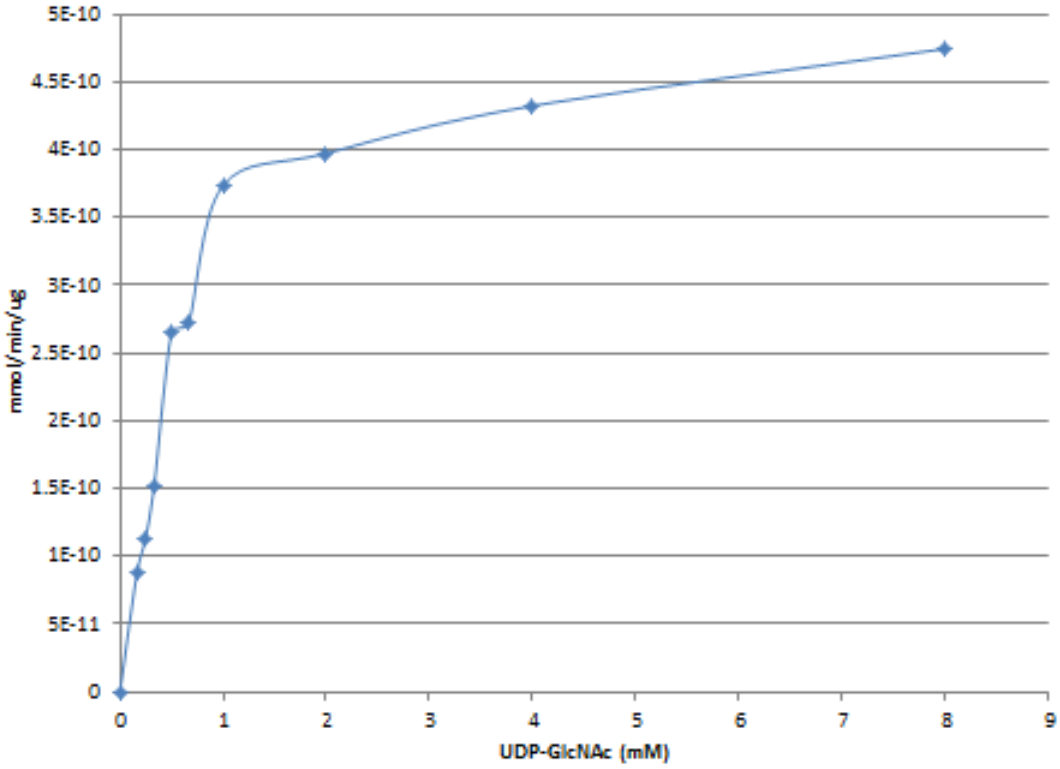


Figure 3-3. Effect of varying enzyme concentration on POMGnT1 rate.
Recombinant POMGnT1 was incubated with known substrates, UDP-GlcNAc and an O-mannose modified peptide, for 4 hours in reaction buffer. Samples contained variable concentrations of POMGnT1, 0-4 ug per reaction. The reaction was neutralized using 1% TFA and the peptide was cleaned up over a C18 reverse phase column, eluted and analyzed via scintillation counting. The reaction rate of UDP-GlcNAc addition per unit POMGnT1 continued to be linear throughout the 4 ug point.

A


5E-10
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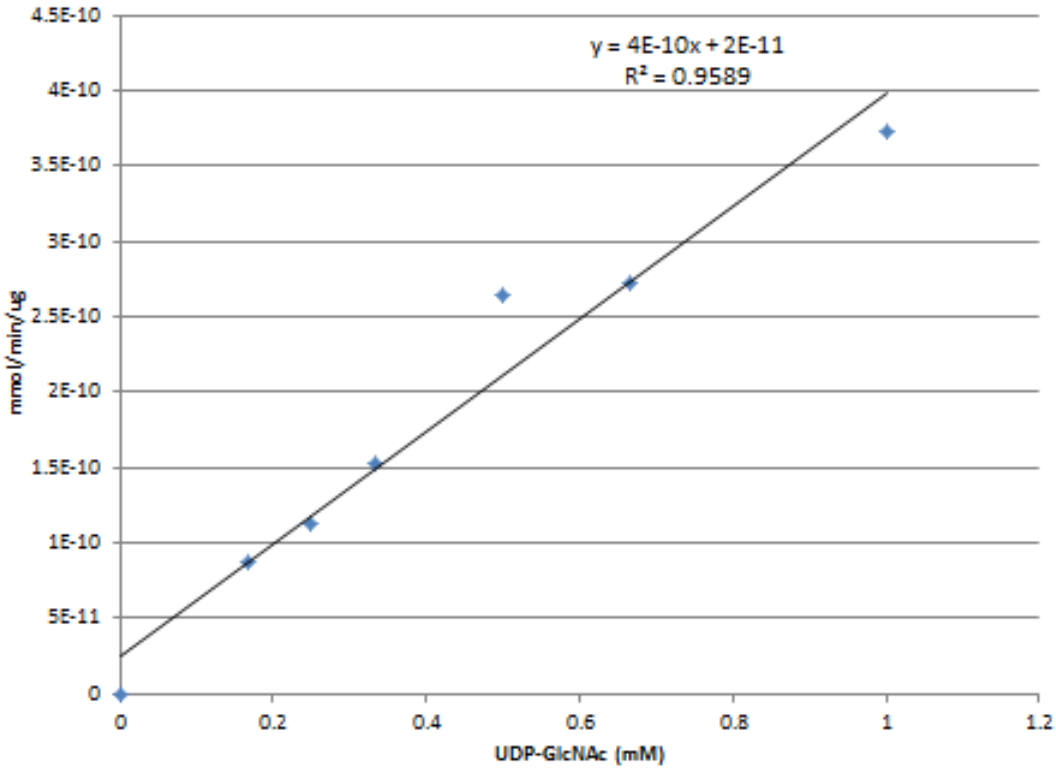
4E-10
3.5E-10
3E-10
2.5E-10
2E-10
1.5E-10
1E-10
5E-11
0
mmol/min/ug
0
1
2
3
4
5
6
7
8
9
UDP-GlcNAc (mM)

B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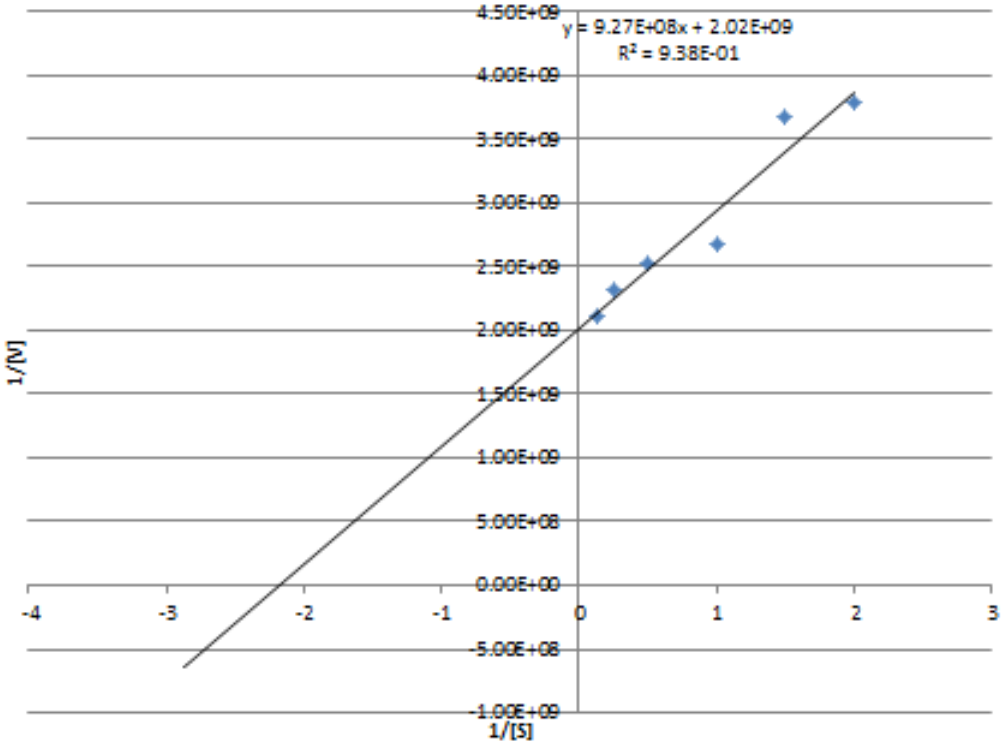
3.5E-10
3E-10
2.5E-10
2E-10
1.5E-10
1E-10
5E-11
0
mmol/min/ug
0
0.2
0.4
0.6
0.8
1
1.2
UDP-GlcNAc (mM)

C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2.50E+09
2.00E+09
1.50E+09
1.00E+09
5.00E+08
0.00E+00
-5.00E+08
-1.00E+09
-4
-3
-2
-1
0
1
2
3
1/[V]
1/[S]

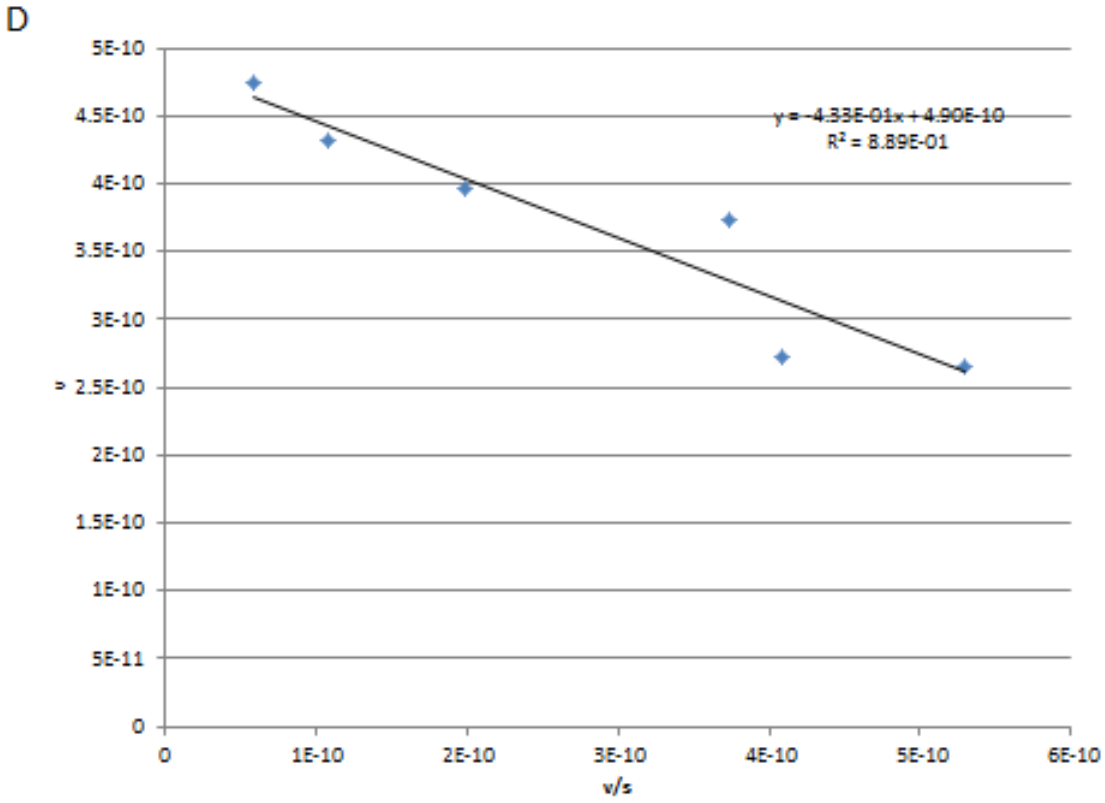
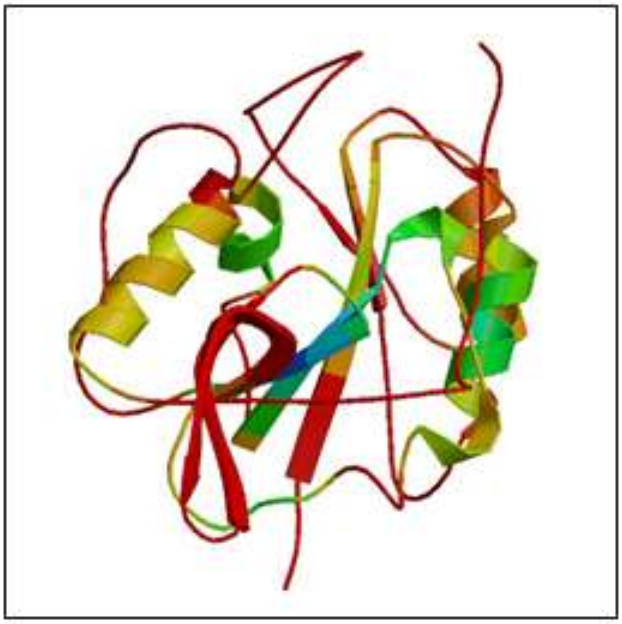


Figure 3-4. Km of recombinant POMGnT1.
(A) Recombinant POMGnT1 (2.5ug constant) was incubated with known substrates, UDP-GlcNAc and an O-mannose modified peptide, for 4 hours in reaction buffer. The amount of UDP-GlcNAc varied per each sample from 0-8 mM. The reaction was neutralized using 1% TFA and the peptide was cleaned up over a C18 reverse phase column, eluted and analyzed via scintillation counting. The reaction rate of POMGnT1 began to approach maximum velocity as the UDP-GlcNAc concentration reached 1 mM. However, (B) the reaction rate of POMGnT1 remained constant between 0 and 1 mM UDP-GlcNAc. (C) When displayed as a Lineweaver-Burk double reciprocal plot the Km of POMGnT1 for UDP-GlcNAc can be ascertained as 0.48 (mM) while the Vmax is 4.9E10 mmol/min/ug POMGnT1. (D) Both the Km and Vmax were additionally confirmed by an Eadie–Hofstee diagram.

A



B

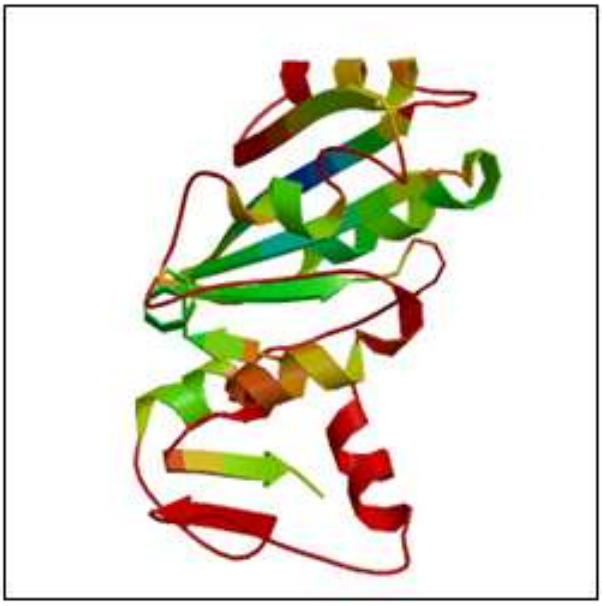


Figure 3-5. SWISS-MODEL homology modeling of rPOMGnT1 mutants suggests change in tertiary structure.
Homology model of rPOMGnT1 based on the known crystal structure of GnT1 (PDB code 1FOA). A majority of the mutants have models comparable to that of WT rPOMGnT1 (A; Supplement), however, the R442C and C490Y mutants have a drastic predicted shift in tertiary and secondary structures (B).

Table 3-1: Radiolabeling Experimental Protocol		
Material	**Stock**	**Per 25ul Reaction**
100 mM Mes (pH 7.0)	1 M (19.5 g in 100mL, pH-NaOH)	2.5ul
10 mM $MnCl_2$	100 mM (1.2584 g in 100 mL)	2.5ul
1 % Triton X	10% Triton X	2.5ul
5 mM AMP	50 mM (17.36 mg in 1 mL)	2.5ul
H2O		25-x
UDP-[3H]GlcNAc	1 mCi/mL: take 10 ul to 500ul 50%ethanol	2.5ul
UDP-GlcNAc	200 mM (26 mg in 200 ul of 100mM MES)	vary
Synthetic O-man peptide	10 ug/ul	vary
Purified Enzyme	1 ug/ul	vary

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to draw conclusions from genotype-phenotype relationships can give insight into yet uncharacterized components of the same gene. By developing an expression system to produce relatively pure rPOMGnT1 with site specific point mutations we were able to produce protein in a manner conducive to downstream catalytic analysis. This approach can be used in further examination of POMGnT1 and other mutations associated with muscle-eye-brain disease. Of the analyzed mutations, two (R442C and C490Y) were not expressed, two were thermal stability mutants (A303V and R605H) and the D556N mutant is a Vmax mutant (Table 4-1).

Future experiments will involve examining the O-man peptide Km of the rPOMGnT1 mutants. Although the UDP-GlcNAc Km was not found to be significant, the peptide substrate Km could have a profound effect on catalytic activity. Additionally, thermal stability of mutants in biologically relevant buffer will give better insight into protein structure and stability. Mutants in this study were examined in Tris, Glycine pH 6.0, and should be analyzed in MES buffer. Finally, to confirm that the expression system used is applicable, R442C and C490Y mutant patient samples should be analyzed for the presence of POMGnT1 protein. Since our R442C and C490Y rPOMGnT1 protein expressed in cell culture, using our expression vector it cannot be concluded that these mutants are definitely not expressed in patient samples.

Table 4-1 Summation of rPOMGnT1 Studies

	Protein Expression	Steady State mRNA Expression	Vmax	UDP-GlcNAc Km	Thermal Stability	Homology Modeling
A303V	+	+	-	+ / -	-	+
R311G	+	+	-	+	+	+
R442C	-	+				-
C490Y	-	+				-
D556N	+	+	+	+	+	+
R605H	+	+	-	+	-	+