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Gain of glycosylation: a new pathomechanism of Myelin Protein Zero mutations

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Abstract

We report the first case of a missense mutation in *MPZ* causing a gain of glycosylation in Myelin Protein Zero (P0), the main protein of peripheral nervous system myelin. The patient was affected by a severe demyelinating neuropathy caused by a missense mutation, D32N, that created a new glycosylation sequence. We confirmed that the mutant protein is hyperglycosylated, is partially retained into the Golgi apparatus *in vitro* and disrupts intercellular adhesion. By sequential experiments, we demonstrated that hyperglycosylation is the main mechanism of this mutation. Gain of glycosylation is a new mechanism in CMT1B.

Introduction

Myelin Protein Zero (P0) is the main structural protein of peripheral myelin¹. P0 consists of three structural domains; an immunoglobulin-like extracellular (ECD), a transmembrane and an intracellular domain. The first 29 amino acids of the protein sequence are cleaved prior to insertion of the protein into the myelin sheath¹ and are not included in the subsequent numbering system of amino acids in this manuscript. P0 is synthesized in the endoplasmic reticulum (ER), glycosylated at asparagine 93 and trafficked through the Golgi Compartment (GC) to the myelin sheath². Here the protein forms a lattice of homotetramers mediated by the ECD within the membrane and interacts with similar structures on the opposing myelin wrap to mediate the compaction of myelin³.

Charcot-Marie-Tooth Type 1B (CMT1B) collectively defines a group of inherited neuropathies caused by mutations in the gene encoding P0 (*MPZ*)⁴. The Inherited Peripheral Neuropathies Database (<http://www.molgen.ua.ac.be/CMTMutations/default.cfm>) lists 126 mutations associated with neuropathy. CMT1B is an autosomal dominantly transmitted disorder though cases often present *de novo*⁵.

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Most *MPZ* mutations are separable into two groups: one causing a severe, early-onset, demyelinating neuropathy and a second, causing a late-onset neuropathy with prominent axonal loss⁵. In a previous paper⁶, we have shown that early and late-onset CMT1B are likely to be caused by multiple pathogenic mechanisms involving gain of function, dominant negative effects and partial loss of function. A toxic gain of function was definitively proved in an animal model carrying the *MPZ* mutation S34del where the mutant protein is retained into the ER and associated to a maladaptive unfolded protein response⁷. We have identified a patient with CMT1B in which the mutant P0 causes hyperglycosylation.

Methods

See supplementary materials.

Results

P0D32N causes severe, early onset CMT1B

The patient is a 46-year-old Italian woman, with no family history of neuromuscular disease (both parents were normal clinically and electrophysiologically). She did not begin walking until two years of age. In the childhood, she fell frequently and was severely impaired in running and climbing. During her second decade she developed prominent wasting and weakness of both hands and legs. Because of severe pes equinovarus she underwent surgery at 24 years of age. On neurological examination she presented bilateral claw hand deformity and foot drop, with moderate proximal wasting and weakness (4/5 MRC) of the lower limbs. Vibration sense was decreased at the lower limbs. Deep tendon reflexes were diffusely absent and a stepping gait was observed. Both motor and sensory action potentials were unrecordable. A sural nerve biopsy, performed when she was 20 years old, showed a severe loss of myelinated fibers with some residual fibers containing myelin outfoldings (Supplementary Fig 1).

By direct sequencing, a transition c.181G>A in *MPZ* gene was demonstrated, producing an amino acid change from aspartic acid to asparagine in position 32 (D32N). The mutation was excluded in 300 normal controls. Mutations in other genes, including *PMP22* and *EGR2*, have been ruled out.

D32N adds a second glycosylation site to P0

The mutation D32N introduces a new asparagine two amino acids before the serine 34, thus creating a new second potential glycosylation motif (NXS/T) in the ECD.

By western blot analysis on transfected HeLa lysates we demonstrated that the molecular weight (MW) of P0D32N was higher compared with the MW of wild type P0 (wtP0), consistent with the increased glycosylation of the mutant protein. To disrupt the novel glycosylation sequence we next generated a double mutant construct P032N+S34A in which the A replaces the required S/T of the NXS/T glycosylation motif. The MW of the double mutant protein returned to the size of wtP0 fusion protein as would be expected if the added glycosylation site was eliminated. To further prove the point we treated P0D32N transfected cells with 0.1 µg of tunicamycin, a drug that inhibits N-linked glycosylation. The treatment reduced the MW of the mutant protein to a size below that of wtP0 and similar to that of P0N93A (Fig 1A), a mutant protein in which the normal glycosylation of P0 is prevented⁸.

The wild type protein, but not P0N93A, could be digested with PNGaseF, an enzyme that removes N-linked carbohydrates. However, the double mutant P0D32N+N93A that eliminates the usual glycosylation site in P0 and is therefore expected to have a unique glycosylation site in position 32 instead of 93, was able to be digested by PNGaseF, as was

wtP0⁶ (Fig 1B). Taken together these results demonstrate that the P0D32N mutation adds a second glycosylation site to the ECD of P0.

Hyperglycosylation impairs intracellular trafficking of P0D32N

To determine the effects of P0D32N on intracellular trafficking we investigated the localization of mutant P0D32N in HeLa cells by confocal microscopy (TCS SL2, Leica) after transient transfection. The mutant protein was partially visualized within the cytoplasm. Alternatively, the wild type counterpart was only observed on the cell surface following similar transfection studies. We found that P0D32N co-distributed with red fluorescent Wheat Germ Agglutinin (WGA), a dye labelling the GC in fixed cells, suggesting that the mutant protein was partially segregated into the GC (Fig 2A to 2C). No co-localization was observed with ER or endosome markers.

Immuno-electron microscopy (EM) also indicated that P0D32N was retained within the GC. HeLa cells expressing wtP0 or P0D32N were doubly labelled with an anti-EGFP antibody and an antibody specific for GM130, a cis-medial Golgi protein. Co-localization of P0D32N and GM130 was evident, while wtP0 was expressed on the surface and did not co-localize with the Golgian protein (Fig 2D and 2E). To ensure that our results were not affected by studies in heterologous cells we repeated immuno-EM in Schwannoma cells. These studies also demonstrated the co-distribution of P0D32N and GM130 (Supplementary Fig 2).

Since glycosylation occurs predominantly in the GC we hypothesized that complications resulting from a second glycosylation site in P0D32N were contributing to the retention of the mutant protein in the GC. To test this hypothesis we performed similar trafficking studies with our P0D32N+S34A construct which has a single glycosylation site although it retains the D32N mutation. The double mutant protein was not retained within the GC and was expressed like wtP0 on the cell membrane. Similarly, P0D32N+N93A which is predicted to have a unique misplaced glycosylated asparagine, also was not retained in the GC and was well expressed on the cellular surface (Fig 3A to 3C).

While P0D32N co-localized with GC markers as shown above, there was also significant amounts of the mutant protein that appeared on the cell surface suggesting that it had been trafficked appropriately out the ER. We wondered whether the additional glycan moieties of the mutant protein might also exert a detrimental effect on the adhesive properties of P0. To test this hypothesis we analyzed aggregate formation in HeLa cells transfected with different cDNA constructs. Both cells expressing P0D32N and the empty vector pEGFP-N1 formed significantly smaller aggregates compared to cells transfected with wtP0 ($51.0\% \pm 5.6\%$; $51.6\% \pm 4.8\%$, $p < 0.0001$), suggesting that the P0D32N expressed into the cell membrane was reducing the adhesive properties of P0. The double mutants P0D32N+S34A and P0D32N+N93A in which the new or the native NXS/T sequon are abolished, formed large aggregates, similar to wtP0 (P0D32N+N93A: $96.7\% \pm 8.80\%$, p : n.s.; P0D32N+S34A: $110.2\% \pm 14.4\%$, p : n.s), suggesting that it was the amount of glycosylation rather than the location of the site that was altering adhesiveness (Fig 3D). Taken together, our results demonstrate that the increased glycosylation in P0D32N contributes to the pathogenesis of the neuropathy.

Discussion

We have a case of a CMT1B in which the D32N missense mutation creates a second glycosylation motif. This mutation causes a severe, early onset, demyelinating neuropathy with myelin outfoldings in the sural nerve biopsy.

We considered three potential mechanisms by which the mutation might cause neuropathy: (1) the substitution of a negatively charged acidic amino acid by a neutral one, (2) the introduction of a site of glycosylation in a wrong position of the molecule and (3) the quantitative increase of sugar chains. By sequential experiments we provided proof of principle that hyperglycosylation is the main mechanism of this mutation.

P0D32N was shown to be partially retained within the GC and partially reaching the cell membrane. While retention into the ER was reported for severe early onset P0 mutants^{6,7} as in many other diseases, a Golgian distribution is rarely reported in human pathology⁹. The partial co-distribution of this mutant with GC markers is strictly associated with hyperglycosylation. It is conceivable that P0D32N may acquire a wrong conformation within the GC, both slowing the trafficking of the mutant protein to the cell surface¹⁰ and decreasing its adhesive properties.

Myelin outfoldings are the pathological hallmark of recessive CMT4B, due to mutations in genes coding for myotubularin related proteins 2 and 13¹¹, but have been also described in several cases of CMT1B^{12,13}. Myelin outfoldings have been recently associated with an impairment of complex pathways regulating myelin thickness in CMT4B¹⁴, but why the D32N or other *MPZ* mutations cause focal misfolding in myelin, is unknown¹².

Gain of glycosylation seems to occur in the 1.4% of human mutations¹⁵, but was never described as a pathomechanism in *MPZ* mutations or in any other myelin-related genes.

With the help of a free, dedicated software, NetNGlyc 1.0 Server¹⁶, we have identified beyond D32N, other three *MPZ* mutations of the ECD potentially able to produce hyperglycosylated forms of P0: D80N¹⁷, D89N¹⁸ and K109N¹⁹. Interestingly, most of those mutations are associated with an early onset, demyelinating phenotype, while the two mutations causing a loss of glycosylation: N93S²⁰ and T95M⁶ are both responsible for a late onset axonal phenotype. This is in keeping with previous data¹⁵, suggesting that loss of glycosylation is generally less severe than hyperglycosylation.

Thus, for P0-neuropathies gain-of-glycosylation could represent a new, significant pathomechanism. Since peripheral myelin is largely composed by glycoproteins²¹, we wonder if hyperglycosylation could be a more general pathomechanism in CMT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Denaturing western blot with a specific antibody for EGFP. The MW expected for the fusion wtP0 EGFP is 56kDa. A) The MW of P0D32N is significantly higher compared with wtP0 (highlighted bands); P0D32N+S34A has a MW comparable to wtP0 because this mutation disrupts the new glycosylation in N32. Moreover, if we treat P0D32N expressing cells with 0.1 μ g of tunicamycin, the MW becomes even lower. P0N93A has a MW lower compared with wtP0 because this mutation is unglycosylated. B) Digestion with PNGaseF. The treatment with PNGaseF decreases the MW of P0D32N (2nd lane) compared with the untreated control (1st lane), while it does not affect the MW of the P0N93A (3rd and 4th lanes), consistent with the hypothesis that the latter mutant corresponds to an unglycosylated form of P0. Interestingly, the double mutant P0D32N+N93A is again sensitive to PNGaseF treatment (5th and 6th lane) thus showing that the new asparagine, in position 32, is glycosylated.

Figure 2.

Trafficking of the mutant P0D32N in HeLa cells after transient transfection. Confocal microscopy (A, B, C) and Immuno-EM (D, E). A) P0D32N expressing cells labelled with red fluorescent Wheat Germ Agglutinin (WGA) which recognizes the GC. B) P0D32N-EGFP is partially expressed on the cell surface and in part intracytoplasmic retained. C) The intracellular part of P0D32N co-distributes with red fluorescent WGA, suggesting that the mutant protein is partially segregated into the GC. D,E) HeLa cells transfected with wtP0 (D) and P0D32N (E) and then doubly labelled with an anti-EGFP antibody, followed by 10nm gold conjugated protein A and an anti-GM130 antibody, followed by 15nm gold conjugated protein A. The wtP0 is expressed on the surface (Fig. 3D, thin arrow) and does not co-localize with GM130 (Fig. 3D, thick arrow). Instead, P0D32N (thin arrow) co-localizes with GM130 (thick arrows) in HeLa cells (Fig. 3E).

Figure 3.

Confocal microscopy (A, B, C) and graphic of adhesion test (D). A) HeLa cells expressing P0D32N. The mutant protein is only partially trafficked to the membrane. B) HeLa cells transfected with P0D32+S34A. This mutant protein is normally expressed on the cellular surface. C) HeLa cells transfected with P0D32N+N93A. The mutant protein is well trafficked to the cell membrane. D) Adhesion test on transfected HeLa cells. Results are from five independent experiments and every measure is normalized to average area of wtP0 aggregates. Cells expressing P0D32N form smaller aggregates than wtP0, like the empty vector (negative control). The double mutants P0D32N+S34A (removing the new glycosylation site) and P0D32N+N93A (removing the native glycosylation site) form larger aggregates, similar to wtP0 ($p < 0.0001$).