Phenotypic Differences between Peripheral Myelin Protein-22 (PMP22) and Myelin Protein Zero (P₀) Mutations Associated with Charcot-Marie-Tooth-Related Diseases

IGOR SHAMES, ANDREW FRASER, PHD, JOSHUA COLBY, WAYEL ORFALI, MD, AND G. JACKSON SNIPES, MD, PHD

Abstract. Mutations in the genes for peripheral myelin protein-22 (PMP22) and myelin protein zero (P₀) cause human hereditary neuropathies with varying clinical and pathological phenotypes. In this study, we examine the effects of representative disease-causing mutations on the subcellular distribution of their corresponding PMP22- and P₀-enhanced green fluorescent protein (EGFP) fusion proteins. In transiently transfected HeLa and 293 cells, we find that wild-type P₀-EGFP and PMP22-EGFP are efficiently synthesized and transported through the secretory pathway to the plasma membrane. The P₀-EGFP and PMP22-EGFP mutants can be classified into several groups: those that are transported to the plasma membrane as in the majority of P₀ mutants; those that are retained in the endoplasmic reticulum as in the majority of PMP22 mutants; and those that are a mixture of the two. In addition, several of these disease-causing mutations are associated with the development of abnormal intracellular cytoplasmic structures that we have previously identified as either intracellular myelin figures or aggresomes. Our studies indicate that different types of PMP22 and P₀ mutations are associated with specific intracellular chaperone proteins, including calnexin and BiP, and that these associations can be altered by glycosylation. These findings indicate that the various P₀ and PMP22 mutants may exert their pathogenic effects in different subcellular compartments and by different mechanisms in the mammalian cell.

Key Words: Calnexin; Endoplasmic reticulum; Myelin; Neuropathy; Peripheral myelin protein-22 (PMP22); Protein zero (P₀).

INTRODUCTION

Mutations affecting a variety of Schwann cell genes cause hereditary peripheral nerve disease characterized by myelin abnormalities (1). Based on disease prevalence, the most significant of these genes encode for relatively abundant protein components of myelin, specifically peripheral myelin protein-22 (PMP22), Protein zero (P₀), and connexin 32 (Cx32). Mutations in each of these genes cause nerve deficits, in part, as a result of myelin loss and reduction of nerve conduction velocities. Among the myelinopathies genes in peripheral nerve, PMP22 (2) and P₀ (3) are localized to compact myelin, whereas Cx32 is found in noncompact myelin (4). PMP22 and P₀ may be functionally related since PMP22 and P₀ are spatially and temporally coexpressed in vivo and in vitro (5). Indeed, recent evidence suggests that PMP22 may oligomerize with itself (6) and with P₀ (7) during myelination.

Protein zero (P₀) is the major structural protein of peripheral nerve myelin, comprising approximately 50% to 70% of total myelin protein. Functionally, P₀ is a transmembrane protein and member of the immunoglobulin gene superfamily (8). The crystal structure has been solved for the extracellular domain of P₀, indicating that P₀ likely functions as a tetrameric homophilic adhesion molecule (9). The basic cytoplasmic domain may serve to stabilize adhesion between the intracellular leaflets of the plasma membrane in myelin, function in signal transduction (10), and mediate interaction with components of the cytoskeleton (11). A large number of disease-causing mutations, including missense, nonsense, and deletion mutations, have been identified in the human gene for P₀ (MPZ). These mutations give rise to several hereditary nerve diseases of varying severity, including Charcot-Marie-Tooth disease (CMT) type 2 (“type 2” denotes a predominantly “axonal” neuropathy), type 1b (“type 1” denotes a “demyelinating” neuropathy; the “b” designation implies linkage to the MPZ gene on human Chr. 1), Dejerine-Sottas syndrome (DSS), and congenital hypomyelinating neuropathy (CHN) (12).

PMP22 is a polytopic membrane glycoprotein that comprises approximately 2% to 5% of myelin protein (13). At present, the function of PMP22 is unknown, although it has been hypothesized to function in cellular adhesion, apoptosis, and signal transduction (14). Duplication of the PMP22 gene located on human chromosome 17p11.2 (15), as well as a number of missense mutations in the PMP22 gene, cause Charcot-Marie-Tooth disease type 1a (CMT1a; the designation “a” implies linkage to the PMP22 gene on Chr. 17). Missense mutations in PMP22 may also cause DSS (16), and hereditary neuropathy with liability to pressure palsies (HNPP; see ref 17). We and others have shown that many PMP22 missense mutations are associated with abnormal retention of the mutant protein in the endoplasmic reticulum (ER) in transfected cells in vitro (18, 19) and following adenoviral delivery of mutant proteins to myelinating Schwann cells in vivo (20), in part, as a consequence of
abnormal interactions with the ER chaperone, calnexin (21). Functional loss of one PMP22 (22) or MPZ (23) allele is sufficient to cause myelinopathy. However, the marked differences in severity caused by different mutations, particularly, the murine Trembler (Tr; G150D) and Trembler-J (Tr-J; L16P) mutations, indicate that many of these mutations act through "gain of function" mechanisms (24).

In summary, a large number of PMP22 and MPZ mutations have been identified as causing hereditary neuropathies characterized by abnormal myelination. We hypothesize that these mutations can cause peripheral nerve disease through a variety of general and Schwann cell-specific cellular mechanisms ranging from abnormal biosynthesis to abnormal processing, abnormal targeting to myelin, and abnormal function in myelin. This hypothesis is supported by the large number of missense mutations that have been identified throughout the MPZ and PMP22 coding regions, presumably differentially affecting functional domains, as well as the marked variability in the severity of their phenotypes (for an updated compendium, see http://molgen-www.uia.ac.be/CMTMutations/). As a first step in categorizing the effects of various mutations and for identifying potentially informative mutations affecting PMP22 and MPZ, we recreated representative disease-causing mutations fused to enhanced green fluorescent protein (EGFP) variants, and examined their intracellular distribution in transfected HeLa and 293 cells.

MATERIALS AND METHODS

All PCR products were generated by the Expand High Fidelity PCR System (Roche, Indianapolis, IN) according to the manufacturer's instructions. All recombinant plasmid constructs were confirmed by automated DNA sequencing. Restriction enzymes were obtained from New England Biolabs (Beverly, MA).

Site-Directed Mutagenesis/Fusion Constructs

Mouse PMP22 and rat P0 cDNAs were used as templates for PCR to incorporate restriction sites for subcloning into pEGFP-N1 or pDsRed2-N1 (BD Bioscience Clontech, Palo Alto, CA) and for the incorporation of a myc epitope tag for P0. The missense mutations were introduced into pEGFP-N1-P0 or pEGFP-N1-PMP22 by site-directed mutagenesis using either the Transfectam (BD Bioscience Clontech) or Quickchange (Stratagene, La Jolla, CA) site-directed mutagenesis kits. All clones were verified by sequence analysis.

Construction of P0-EGFP Expressing Adenovirus

We constructed a recombinant adenovirus for P0-EGFP expression by homologous recombination in pBJS183 E. coli (25). Briefly, P0-pEGFP-N1 was digested with Nsi I and Afl II to release the expression cassette containing the 5' cytomegalovirus promoter, the P0-EGFP coding region, and a 3' SV40 poly A tail that was ligated into the shuttle vector (pShuttle; see ref 34) that was recombined with the cosmid pAdEasy-1, as described previously (20).

Cell Culture and Adenoviral Infections

Primary human embryonic kidney cells (QBI-293A; Qbiogene, Carlsbad, CA) and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM; Bio-Whittaker, Walkersville, MD), supplemented with 10% bovine calf serum (HyClone, Logan, UT), 2 mM glutamine (Invitrogen Life Technologies, Carlsbad, CA), 1% Antibiotic/Antimycotic (Invitrogen Life Technologies) at 37°C in a humidified incubator containing 5% CO2. 293A cells were infected with adenovirus in supplemented DMEM for 90 min at 37°C.

Recombinant Adenovirus Administration and Tissue Preparation

Recombinant adenovirus was expanded, purified, quantitated, and injected into sciatic nerves as described previously (20). Fourteen days after injection, animals were euthanized with 15 mg ketamine (i.p.; Wyeth-Ayerst, Madison, NJ), and the sciatic nerves were removed and fixed in 4% paraformaldehyde/PBS at 4°C for 3 hours (h). Nerves were cryoprotected overnight at 4°C in 30% sucrose, embedded in OCT (Tissue-Tek, Sakura, Torrence, CA) and frozen in isobutanol/dry ice (−30°C). Ten-μm-thick frozen sections of the rat sciatic nerves were prepared using an HM 500M cryostat (Microm, Waldorf, Germany) and stored at −80°C.

Immunofluorescence: Tissue Sections

Tissue cryosections were immersion fixed in 4% paraformaldehyde/PBS (20 mM NaH2PO4, 150 mM NaCl) for 10 min, and immunostained as described (26) with the following modifications: primary and secondary antibodies were diluted in a blocking buffer consisting of PBS/0.2% Tween-20/10% milk. Mouse monoclonal anti-MBP antibody (1:300; Roche, Indianapolis, IN) was used as a primary antibody and Cy3-conjugated goat anti-mouse antibody (1:500; Jackson Immunoresearch, West Grove, PA) was used as a secondary antibody. In some sections, nuclei were stained with Hoechst dye 33258 (Pierce, Rockford, IL) diluted 1:50,000 in PBS/0.2% Tween-20 for 15 min. Sections were mounted with Immuno Floures Mounting Media (ICN, Aurora, OH). Immunostained sections were visualized by laser-scanning confocal microscopy (LSM 410; Zeiss, Göttingen, Germany).

Transfection and Immunostaining

Sixty-thousand HeLa (or 293) cells were plated on 12-mm-diameter coverslips, incubated in media for 24 h and transfected with 2 μg of expression plasmid DNA using calcium phosphate precipitation. Fresh media was added 24 h after transfection, cells were incubated for 48 h, rinsed twice with warm PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.2), and fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. The cells were rinsed twice with PBS/0.2% Triton X-100 (ICN), blocked with PBS, 0.2% Triton X-100, 5% normal goat serum, 2% bovine serum albumin for 30 min, incubated with either mouse monoclonal anti-Golgi 58K protein (Clone 58K-9, 1:1,000; Sigma-Aldrich, St. Louis, MO), anti-myc (clone 9E10, Covance, Berkeley, CA), anti-calnexin

Fig. 1. Schematic diagram of PMP22 and P₀ with the position of representative disease-causing mutations examined in this study along with their associated phenotype (squares) and subcellular localizations (circles). The exact mutations examined are summarized in the Table. Note that most of the PMP22 disease-causing mutations are associated with an ER-like localization, whereas most P₀ disease-causing mutations are associated with a plasmalemmal localization. The structure of PMP22 is taken as one of the alternatives described in reference 36. TM1/HD1 refers to the first transmembrane domain (TM)/hydrophobic domain (HD) relative to the N-terminus of PMP22. The branching lines represent sites of N-glycosylation.

(1:1,000, Stressgen, Victoria, BC, Canada), anti-BiP (1:1,000, Stressgen) or mouse monoclonal anti-protein disulfide isomerase (anti-PDI) (Clone RL 77; 1:1,000; Affinity Bioreagents, Golden, CO) for 1 h in blocking solution, followed by 3 washes in PBS/0.2% Triton X-100. The cells were incubated with Cy3-conjugated goat anti-mouse or anti-rabbit antibody (1:1,000; Jackson Immunoresearch) for 1 h in blocking solution, washed 3 times in PBS/0.2% Triton X-100, and mounted with Immuno Floures Mounting Media (ICN). For visualizing lysosomes, transfected cells were treated with Lysotracker Red DND-99 (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Fluorescently labeled cells were visualized by standard fluorescence microscopy (Axioskop 2; Zeiss) or by confocal microscopy (LSM 410; Zeiss).

RESULTS
Validation of PMP22-EGFP and P₀-EGFP Constructs

Our experimental strategy was to use P₀- and PMP22-EGFP fusion proteins to visualize the effects of individual disease-causing MPZ and PMP22 mutations on their intracellular processing as an initial screen for genotype/phenotype correlations (Fig. 1). First, we had to establish whether the addition of the EGFP to the carboxy terminus of PMP22 and P₀ interfered with the trafficking of the fusion proteins. Our previous studies employing a vesicular stomatitis virus epitope tag at the carboxy-terminus of PMP22 demonstrated that the position of this modification did not interfere with the trafficking of PMP22 delivered by adenovirus to the myelin sheath of Schwann cells in vivo (20). In further control experiments, we verified that HeLa cells transfected with PMP22wt-EGFP expressed the fusion protein on the cell surface, whereas cells transfected with PMP22Tr-J-EGFP and PMP22Tr-EGFP retained the fusion proteins in the ER, as expected from previous studies (18–20) on non-EGFP tagged wild-type and mutant PMP22 proteins. Thus, PMP22-EGFP fusion proteins appear to be useful reagents for investigating the trafficking of wild-type and mutant PMP22 proteins.
membrane staining (Fig. 3). Thus, we conclude that the P0 fusion protein is efficiently synthesized and can be incorporated into myelin-like profiles in Schwann cells in vivo and is efficiently transported through the secretory pathway in HeLa cells in vitro.

**Generation and Characterization of P0-EGFP and PMP22-EGFP Mutants Representing Human Hereditary Neuropathies**

Next, we selected representative PMP22 and P0 mutations that have been shown to cause human hereditary neuropathies (Table). The mutations were chosen to represent different hereditary neuropathy phenotypes (CMT2, CMT1, DSS, HNPP, and CHN) and to cover the major structural domains of P22 and P0 (intracellular, extracellular, transmembrane, glycosylation) as shown schematically in Figure 1. For convenience, Figure 1 and the Table also provide an overview of our transfection studies using mutant P22- and P0-EGFP fusion constructs. We found that most P22 and P0 mutations could be placed in the following categories: 1) normal transport to the plasma membrane, 2) ER retention, 3) combined ER retention and transport to the plasma membrane, 4) ER, Golgi, and plasma membrane. Interestingly, we found major differences between the effects of P0 mutations and PMP22 mutations.

**The Majority of Disease-Causing PMP22 Mutations Are Retained in the Endoplasmic Reticulum**

In our studies, the following PMP22 mutations caused abnormal retention of the PMP22-EGFP fusion protein in the ER: G150D (Trembler), L16P (Trembler-J), L19P, D37V, S76I, S79C, G107V, and G150C (representative colocalizations with organelle-specific markers are shown in Fig. 3). By immunofluorescence, the subcellular localization of the vast majority of these disease-causing PMP22 missense mutations were characterized by colocalization of the PMP22-EGFP fusion protein with the ER marker, PDI, and did not significantly overlap with Golgi 58k immunostaining or show significant fluorescence at the plasma membrane. We discovered 2 exceptions to this generalization, the PMP22-G93R mutation and the PMP22-V30M mutation. The distribution of PMP22-G93R was indistinguishable from wild-type PMP22, whereas PMP22-V30M showed significant colocalization with PDI, but also was expressed on the cell surface, particularly at the interface between adjacent cells (Fig. 3h–j).

Many of the cells transfected with wild-type and mutant PMP22-EGFP exhibited intracellular fluorescence in addition to the ER-like distribution that consisted of EGFP-positive intracellular inclusions that did not stain for PDI. Our previous work (21) indicated that these inclusions are likely to be intermediates targeted for protein degradation while retaining their association with the ER resident chaperone, calnexin. To examine this in

![Fig. 2. The P0-wt-EGFP fusion protein is incorporated into myelin-like profiles in Schwann cells in vivo. Sciatic nerves of P10 rats were injected with P0-wt-EGFP recombinant adenovirus. Excised nerves were analyzed by double-label confocal microscopy using indirect immunofluorescence for MBP (Cy3, red) and direct visualization of the P0 fusion protein (green). P0-wt-EGFP demonstrates a myelin-like distribution in a subset of the infected cells (arrows), colocalizing with MBP. Each successive horizontal panel represents a 1-μm-thick optical section in the Z-plane by confocal microscopy. Scale bar = 10 μm.](image-url)
Fig. 3. Subcellular localizations of mutated PMP22 proteins in HeLa (a–d, f–i) and 293A cells (e, j, k). HeLa cells transiently transfected with PMP22-wt-EGFP (a–d) demonstrate plasmalemmal distribution of EGFP (a, c, arrows) with faint colocalization with Golgi 58K and Lysotracker. Because of the extensive surface staining with PMP22wt-EGFP, colocalization with PDI is equivocal (c). 293 cells transfected with PMP22G107V show extensive colocalization with calnexin (CNX) in the reticular ER-like structures as well as in intracellular aggregates (e, arrows). In HeLa cells, PMP22Tr-GFP is extensively colocalized with CNX in a reticular ER-like distribution (f), but PMP22Tr-EGFP (green) does not colocalize with P20-wt-RFP (red) that is extensively expressed on the plasma membrane of HeLa cells (g, arrows). HeLa cells transfected with PMP22V30M-EGFP show extensive colocalization with PDI in an ER-like reticular distribution except in intracellular aggregates (h, arrows) in some transfected HeLa cells. Other HeLa cells clearly demonstrate that PMP22V30M-EGFP can be localized to plasmalemmal sites of intercellular contact between adjacent transfected cells (i, arrows). 293A cells transfected with mutant PMP22-EGFP (V30M, panel j; S76I, panel k, and G107V, panel e) form intracellular aggregates that colocalize with CNX (arrows). Scale bar = 5 μm.

More detail, we transfected 293A cells with PMP22-V30M-EGFP, PMP22-Tr-EGFP, PMP22-TrJ-EGFP, and PMP22wt-EGFP and examined them by double-label immunofluorescence for calnexin. As shown in Figures 3 and 4 for the constructs PMP22-V30M-EGFP, PMP22-S76I-EGFP, PMP22-G107V-EGFP, and PMP22-L16P (Tr-J)-EGFP, the intracellular aggregates colocalized with calnexin, but not PDI (not shown here for
TABLE
Summary of Mutations

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Summary of PMP22 and P0 disease-causing mutations, including the relative position mutated, the subcellular localization of the EGFP fusion protein, and the human disease phenotype (if any) associated with the representative mutations. Individual references to these mutations can be found at http://molgen-www.uia.ac.be/CMTMutations/. Numbering for the P0 amino acids corresponds to the mature P0 protein. Thus, the numbering for P0 provided here differs by 29 amino acids from the numbering provided by the mutation database for inherited peripheral neuropathies cited above. This difference corresponds to the size of signal sequence at the amino terminus of P0 that is cleaved during P0 biosynthesis. Abbreviations: TM, transmembrane; EC, extracellular loop; N-glyc, N-glycosylation site; HD, hydrophobic domain; ER, endoplasmic reticulum; PM, plasma membrane; DSS, Dejerine-Sottas syndrome; CMT, Charcot-Marie-Tooth; CHN, congenital hypomyelinating neuropathy; HNPP, hereditary neuropathy with liability to pressure palsies.

PMP22S107V and PMP22S76I, but see Fig. 4 for PMP22Tr and PMP22Tr-J).

Effect of N-Linked Glycosylation in the ER Retention of Mutant PMP22

Since the ER chaperone protein calnexin is known to bind to newly-synthesized proteins via their asparagine-linked monoglycosylated oligosaccharides through a lectin-like interaction (28), we investigated the importance of the N-linked glycosylation on ER retention of PMP22. First, we created the construct PMP22-N41A-EGFP (PMP22 is glycosylated at N41), which eliminates the N-linked glycosylation site, and then tested whether this mutation would interfere with the trafficking of the glycosylation-deficient PMP22 to the plasma membrane. When transfected into HeLa cells, we found that PMP22 N41A-EGFP is expressed on the cell surface (Fig. 4g–i), like the wild-type protein (Fig. 4a–c), indicating that association with calnexin is not an absolute requirement for PMP22 to successfully traverse the secretory pathway. We then tested whether abolishing the association between mutant PMP22 and calnexin might ameliorate the ER retention of mutant PMP22. Thus, we created the double mutant, PMP22-Tr-J/N41A-EGFP, and tested whether this protein was expressed on the plasma membrane in transient transfection assays. As shown in Figure 4j–l, abolishing the N-glycosylation site did not alter the intracellular distribution of PMP22Tr-J N41A to the ER compartment in transfected 293A cells. Specifically, no PMP22Tr-J/N41A was detected on the cell surface. Abolishing the PMP22 glycosylation site (N41) in PMP22-Tr-J EGFP did, as expected, abrogate the colocalization of mutant PMP22Tr-J-EGFP with calnexin in the abnormal intracellular accumulations in the transfected 293A cells. Despite the fact that PMP22-Tr-J/N41A was no longer bound to calnexin within the abnormal intracellular aggregates, we had the strong impression that the cells transfected with the PMP22-Tr-J/N41A-EGFP double mutant still contained a significant number of the abnormal intracellular aggregates. This suggested that other
The Majority of Disease-Causing P0 Mutations Are Expressed on the Plasma Membrane

Fluorescence microscopy for the P0-EGFP protein carrying the disease-causing P0 mutations S15F, S34C, S34F, S49L, N93S, T95M, K101R, T114M, G138R, Y152stop, or Q186stop revealed that all were transported through the secretory pathway to the plasma membrane. HeLa cells transfected with P0wt-N41A-EGFP and P0-Tr-J/N41A-EGFP abrogated P0/calnexin interactions, and the intracellular aggregates were associated with BiP, but not calnexin (Fig. 4g–l). In no case did overexpression of any of the 4 P0 constructs lead to an association of the EGFP-tagged P0 with PDI. Thus, P0-Tr-J/N41A, in the absence of a significant interaction with calnexin, is likely to be retained in the ER via its interaction with BiP as a backup mechanism for the ER quality control of P0 biosynthesis.

A Subset of Disease-Causing P0 Mutations Are Associated with ER Retention

Two disease-causing P0 mutations had clearly abnormal subcellular distributions: G138R and S34del. By immunofluorescence, both the G138R and the S34del mutation extensively colocalized with the ER markers PDI and calnexin (Fig. 5f–i, k, l). The distribution of the P0,G138R-EGFP molecule, however, differed from the P0,S34del-EGFP in that the former was also expressed on the cell surface at low but detectable levels in a subset of the transiently transfected cells (Fig. 5g, i).

We observed that wild-type and mutant P0-EGFP, like PMP22, was associated with the accumulation of abnormal intracellular structures. We determined the percentage of cells that contained the abnormal intracellular inclusions that had been transfected with representative wild-type and mutant P0-EGFP constructs. These studies showed that P0,G138R-EGFP was associated with significantly more abnormal inclusions than wild-type P0-EGFP, P0,T95M, P0,Y152stop, and P0,Q186stop (Fig. 6). We examined whether the abnormal intracellular inclusions were associated with calnexin by double-label indirect immunofluorescence microscopy. As shown in Figure 5, P0,G138R-EGFP forms fluorescent intracellular aggregates that do not react with anti-PDI or anti-calnexin (Fig. 5g, h). However, intracellular accumulations of P0,S34del-EGFP do show significant colocalization with both calnexin and PDI (Fig. 5k, l). These findings are in contrast to the results obtained above for mutant PMP22 where the abnormal intracellular inclusions in cells transfected with most of the PMP22 disease-causing mutations are associated with calnexin, but not PDI. We conclude that ER retention of proteins carrying disease-causing P0 and PMP22 mutations occurs through distinguishable mechanisms involving ER chaperones.

PMP22 and P0 Colocalize Throughout the Secretory Pathway

Though controversial (29), studies by D’Urso et al (7) indicate that PMP22 and P0 oligomerize. Whereas wild-type and mutant PMP22 associate with calnexin, only certain P0 mutants (e.g. S34del) show this association, suggesting that P0 and PMP22 have different folding interactions in the ER. To investigate whether PMP22 and P0 have similar or different localizations in the secretory pathway, we cotransfected wild-type PMP22-EGFP and P0-myc and found them to be extensively colocalized throughout the cell (Fig. 7a). Thus, there was no evidence that PMP22 and P0 are sorted into separate compartments or are differentially degraded.

PMP22 Mutations Do Not Interfere with Trafficking of P0 to the Plasma Membrane and Visa Versa

The extensive colocalization of PMP22 and P0 in transfected HeLa cells allows us to test whether PMP22 trafficking mutations exert a dominant effect on P0 transport as might be predicted if mutant PMP22, which is retained in the ER, was to bind to and retain wild-type P0 in that compartment. To examine this, we separately cotransfected PMP22wt-EGFP, PMP22-Tr-J-EGFP, and PMP22-Tr-J-EGFP with P0wt with a carboxy terminal myc epitope tag (P0wt-myc) and evaluated for redistribution of the P0 in the presence PMP22 carrying disease-causing mutations. As shown in Figure 7, the presence of these representative PMP22 mutations in the ER had no effect on the trafficking of the tagged P0 protein to the plasma membrane.

There is crystallographic and physical evidence that P0 oligomerizes with itself (9). This raises the possibility that P0 ER retention mutations (P0,S34del and P0,G138R) might have a dominant effect on trafficking of wild-type


P₀ to the cell surface. To test this, we separately cotransfected P₀S34del-EGFP, P₀G138R-EGFP, and P₀wt-EGFP with P₀wt-myc into HeLa cells. Double-label fluorescence microscopy showed that the presence of disease-causing mutations of P₀ (S34del and G138R) did not affect trafficking of P₀wt-myc to the cell surface in HeLa cells (Fig. 7) as detected by indirect immunofluorescence. We did note that the P₀S34del and P₀G138R mutants formed intracellular aggregates that did not colocalize with P₀wt-myc, suggesting that they are sequestered from wild-type P₀ in secretory or disposal pathways. We also investigated whether disease-causing mutations that deleted the intracellular domain of P₀ (Y152stop and Q186stop) might interfere with the trafficking of P₀wt-myc. Thus, we cotransfected P₀wt-myc with P₀−Y152stop-EGFP or P₀−Q186stop into HeLa cells. Double-label immunofluorescence microscopy using Cy3 to label the myc tagged proteins revealed that wild-type P₀ and P₀−Y152stop and P₀−Q186stop mutations strongly colocalize (Fig. 7).

**DISCUSSION**

Mutations in the MPZ and PMP22 genes cause similar pathologies characterized by abnormal myelination and variable axon loss. Mutations in PMP22 and MPZ are pleiotropic and have been associated with the CMT1 and DSS. In general, mutations in PMP22 that cause DSS or CMT are dominant “gain of function” mutations, whereas HNPP results from loss of 1 PMP22 allele (24). Similarly, dominant MPZ mutations that cause CHN or DSS are generally considered “gain of function” mutations, whereas classic CMT1b is often considered as due to a loss of an MPZ allele (12). Indeed, mice heterozygous for a MPZ null allele develop late-onset (adult) neuropathy (23). At present, P₀ and PMP22 are the only known transmembrane proteins that are localized specifically to compact myelin in the peripheral nervous system. Alterations in the dosage of either protein can cause peripheral nerve disease characterized by myelinopathy. The time-course of expression for MPZ and PMP22 mRNAs and proteins during myelination in vivo and in myelinating neuron/Schwann cell cultures are nearly identical and differ from those of other myelin proteins such as MBP and MAG (5). Finally, a variety of studies suggest that P₀ interacts with itself (30), P₀ interacts with PMP22 (7), and PMP22 can associate with itself (31) in myelin. Thus, it is of considerable interest to determine whether PMP22 and MPZ share common pathomechanisms for causing peripheral nerve disease.

There are, of course, significant differences between PMP22 and P₀. PMP22 is a relatively small (22 kDa) polytopic hydrophobic protein. Over 60% of the amino acids in PMP22 reside in hydrophobic domains, probably membrane-associated. The majority of newly-synthesized PMP22 is rapidly degraded from the ER compartment (32). PMP22 may be labile because it is difficult to fold correctly as a consequence of its marked hydrophobicity. P₀ is a larger protein (28 kDa), with a single transmembrane domain (8). Unlike PMP22, the majority of newly-synthesized P₀ protein rapidly traverses the secretory pathway and is stably expressed on the plasma membrane (32). There is approximately 10 to 20 times more P₀ than PMP22 in the myelin sheath (13), although both are present in large quantities relative to other Schwann cell proteins owing to the multiple lamination of myelin membranes. As a first step in elucidating the effects of various MPZ and PMP22 mutations, we determined which cellular compartments are accessed by these mutant proteins.

In our studies, only three PMP22 mutant proteins were transported to the plasma membrane of HeLa cells. These three PMP22 mutants have missense mutations in hydrophilic regions of PMP22 protein (PMP22 N41A, PMP22 V30M, and PMP22 G93R). Of these, the V30M mutation is clearly associated with an HNPP phenotype (N41A is an experimental construct for this study), whereas G93R mutation causes CMT1. The remainder of the disease-causing PMP22 mutations, including all mutations that affect the hydrophobic domains (originally predicted to be transmembrane domains), were associated with ER retention. The PMP22 mutations examined in this study partially overlap with those described by Naef and Suter (19), and those that are duplicated are in agreement with their findings. Thus, the ER retention of PMP22 carrying the L19P, D37V, and G150C mutations can be added to the growing list of PMP22 mutations that are associated with ER retention. It is most likely that integrity of hydrophobic domains of PMP22 protein is essential for proper folding, processing, and insertion into plasma membranes.

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**Fig. 4.** Abolishing the N-glycosylation site in PMP22 does not alter the ER-like localization of PMP22-Tr-J-EGFP, but does alter its association with ER-resident chaperones in intracellular aggregates. 293A cells were transiently transfected with PMP22-wt-EGFP (a–e), PMP22-Tr-J-EGFP (d–f), PMP22-N41A-EGFP (g–i), or PMP22-Tr-J/N41A-EGFP (j–l). The transfected 293A cells were immunostained for the ER quality control/chaperone proteins: calnexin (CNX) (a, d, g, j), BiP (b, e, h, k), and PDI (c, f, i, l). The intracellular aggregates formed by overexpression of PMP22wt-EGFP (a–c, arrows) and PMP22-Tr-J-GFP (d–f, arrows) colocalize with CNX, but not BiP and PDI. Conversely, the intracellular aggregates formed by PMP22-N41A-EGFP (g–i, arrows) and PMP22-Tr-J/N41A-EGFP (j–l, arrows) show significant colocalization with BiP, but not PDI and CNX. Scale bar = 5 μm.
Fig. 5. The wild-type P₀ (a–c) and the majority of mutated P₀ proteins are expressed on the plasma membrane. Intracellular compartments were immunolabeled with mouse anti-PDI and rabbit anti-calnexin (CNX) for ER, and monoclonal-anti-58K marker for Golgi. HeLa cells were transiently transfected with P₀-wt-EGFP (a, b). Note the green fluorescent labeling at the cell surface (a, arrows). In HeLa cells transfected with P₀-N93S-EGFP (d), again note the extension of the green fluorescent staining to the plasma membrane, particularly at sites of intercellular contact (arrows). Some HeLa cells transfected with P₀-S34C-EGFP (e) showed colocalization of the mutated P₀ with the Golgi 58K marker. HeLa cells transfected with P₀-S34del-EGFP (S34d; panel...
Fig. 6. Counts of intracellular aggregates in HeLa cells transiently transfected with wild-type and mutated P_0 constructs. HeLa cells were transiently transfected with P_0-wt-EGFP, P_0-T95M-EGFP, P_0-G138R-EGFP, P_0-Y152stop-EGFP, or P_0-Q186stop-EGFP. Experiments were performed in triplicate. For each transfection, 100 cells were selected at random and assessed for the presence of intracellular EGFP-containing aggregates. The amount of cells with intracellular aggregates is expressed as a percentage of the total number of cells counted. The construct P_0-G138R-EGFP (*), when transfected into HeLa cells, was associated with a significantly higher percentage of cells containing intracellular aggregates when compared to P_0-wt-EGFP any of the other P_0 mutants by 2-way ANOVA (* p < 0.03, ** p < 0.002). None of the other P_0 mutants were significantly different from P_0-wt in this assay.

membrane. In correlating PMP22 genotype with phenotype, we note that all of the PMP22 mutations associated with ER retention cause either a CMT1a or a DSS phenotype. Both of these hereditary neuropahties are characterized as demyelinating/dysmyelinating, with slow nerve conduction velocities by electrophysiology and myelin loss, “onion bulb” formation, and variable axon loss by microscopy. The main difference between CMT1 and DSS is the degree of the disease phenotype; DSS has an earlier onset and more severe electrophysiological and pathological changes associated with myelin loss compared to CMT1. We have not observed any difference in the localization of the mutant PMP22 proteins that correlate with DSS or CMT.

As previously discussed, PMP22 mutations that cause CMT1 or DSS are considered “gain of function” PMP22 mutations in that their phenotype is clearly distinguishable from the “loss of function” PMP22 mutations that are modeled by humans with HNPP that carry an intrachromosomal deletion of chromosome 17p11.2–12 and in heterozygous Pmp22 null mice, which also have a “tomaculous neuropathy,” analogous to HNPP. Thus, it is likely that the “gain of function” associated with the more severe CMT1 and DSS phenotypes resides either in the ER (since these are ER retention mutations) or in degradative pathways associated with removal of mutant proteins from the ER. Recently, we have identified 2 possible ways in which PMP22 interactions with the ER chaperone protein calnexin may alter ER or degradative pathways (21). First, we have demonstrated that mutant PMP22 has a prolonged association with calnexin and that when either wild-type or mutant PMP22 is overexpressed, it associates with calnexin in abnormal intracellular myelin-like structures (thought to be pre-lysosomal) and aggresomes (which accumulate when the proteasome is overloaded). All of the work presented here suggests that the abnormal associations with calnexin are likely to be a common effect of PMP22 ER retention mutations. In addition, we have shown for a representative mutation (PMP22 L16P), that abrogation of the association of mutant PMP22 with calnexin causes the mutant PMP22 to associate with another ER chaperone, BiP. This demonstrates the importance of redundancy in the quality control functions of the ER, at least for glycosylated transmembrane proteins like PMP22.

As noted above, the subcellular localization of the PMP22V30M and the G93R mutations differed from the majority of the PMP22 disease-causing mutations in that the mutant protein could be detected on the plasma membrane, albeit at low levels for V30M. The PMP22V30M and G93R mutations, unlike many of the PMP22 missense mutations, are found in the extracellular domain, not in a region of high hydrophobicity. The PMP22V30M mutation is associated with an HNPP phenotype, thus acting as a loss of function mutation. One interpretation of this result is that V30M and G93R directly interfere with the function of the PMP22 protein. If so, that would make them unique (for now) among the PMP22 missense mutations and suggests that the portion of the PMP22 molecule adjacent to the transmembrane domains is critically important for its function. On the other hand, whereas the
Fig. 7. PMP22 and P₀ mutations do not interfere with trafficking of wild-type P₀ to the plasma membrane. HeLa cells were transiently cotransfected with P₀-wt-EGFP and P₀-wt-myc (a), P₀-Y152stop-EGFP (Y152s) and P₀-wt-myc (b), P₀-S34del-EGFP and P₀-wt-myc (S34d; panel e), P₀-G138R-EGFP and P₀-wt-myc (d), P₀-Q186stop-EGFP and P₀-wt-myc (e), PMP22-wt-EGFP and P₀-wt-myc (f), PMP22-σEGFP and P₀-wt-myc (g), and PMP22-αEGFP and P₀-wt-myc (h). The P₀-wt-myc was visualized by indirect immunofluorescence using a monoclonal anti-myc antibody and a Cy3-conjugated second antibody (red). Note the extensive colocalization of P₀-wt-myc with P₀-wt-EGFP (a), P₀-S34del-EGFP (S34d; panel e), P₀-G138R-EGFP (d), and P₀-Q186stop-EGFP (Q186s; panel e). The presence of P₀-wt-myc on the plasma membrane is unaffected by the presence of P₀-S34del-EGFP (S34d; panel e), P₀-G138R-EGFP (d), PMP22-σEGFP (g), or PMP22-αEGFP (h). For example, when P₀-wt-myc (red) is cotransfected with PMP22-αEGFP (green, panel h) the red fluorescent signal extends beyond the green fluorescent signal (arrow). Also note that the green fluorescent intracellular aggregates formed by P₀-S34del (S34d; panel e, arrows), G138R (d, arrows), PMP22-σ (g, arrows) do not colocalize with P₀-wt myc. Scale bar = 10 μm.

HNPP phenotype associated with the PMP22V30M mutation indicates a loss of function, its partial colocalization with ER markers and association with calnexin in the abnormal intracellular aggregates have been associated with the gain of function in CMT1 and DSS. It may be that the “gain of function” is partially compensated by the fact that PMP22V30M is transported to the plasma membrane. Further studies are indicated to determine the effects of the PMP22V30M and G93R, as we anticipate that identifying disease-causing PMP22 mutations that are not retained in the ER may highlight critical amino acid residues that are required for the function of the PMP22 protein.

The majority of P₀ protein mutants that get transported to the plasma membrane are those with mutations in extra- or intracellular regions of the molecule. A nonconservative missense mutation in hydrophobic transmembrane region of P₀ protein (G138R) interferes with transport of the protein to the plasma membrane causing retention of most of the mutant P₀ in the ER with only a small amount being transported to the plasma membrane. This observation confirms that conservation of hydrophobic
transmembrane domains is crucial for proper intracellular processing and correct plasma membrane insertion in both P\(_0\) and PMP22 proteins. Another \(P_0\) ER retention mutant is \(P_0\)S34del, which carries a mutation in the extracellular hydrophobic immunoglobulin-like domain of \(P_0\) protein. Based on the crystal structure of the extracellular domain of \(P_0\) and computer models, deletion of Ser34 is predicted to alter the \(\beta\)-strand conformation (to favor \(\alpha\)-helix formation) in this region of the \(P_0\) molecule, thereby affecting its stability, normal processing, and localization (9). This hypothesis is consistent with our results that the \(P_0\)S34del is retained in the ER suggesting that \(P_0\)S34del is an unstable molecule. This hypothesis is further supported by the fact that other missense mutations at position S34 (e.g. \(P_0\)S34F and \(P_0\)S34C mutations) are not retained in the ER and are predicted to retain the \(\beta\)-strand conformation of \(P_0\) in this region (modeling strategy described in ref. 33). The \(P_0\)S34C and \(P_0\)S34F mutations may interfere with the adhesive properties of \(P_0\). Based on the packing of the extracellular domain of \(P_0\) in its crystalline form, Ser34 is near a site that is predicted to be important for tetramer formation (9, 12). We anticipated that \(P_0\)S34C would form intracellular aggregates due to the presence of an unpaired cysteine, but the mutant \(P_0\)S34C protein was clearly expressed on the cell surface, though in some cells it partially colocalized with the Golgi marker.

The cytoplasmic domain of \(P_0\) protein is involved in myelin compaction at the major dense line, whereas extracellular domain is involved with myelin compaction at the intraperiod line (8, 34). Moreover, Wong and Filbin (11) demonstrated that the adhesive properties of \(P_0\) are dependent on the presence of cytoplasmic domains. Detergent extraction studies suggested that the intracellular domain of \(P_0\) might be involved in interactions with components of the cytoskeleton (11). From our transfection studies we observed that partial (\(P_0\)-Q186stop) or complete (\(P_0\)-Y152stop) deletion of intracellular domain of \(P_0\) protein does not interfere with the ability of the remainder of the \(P_0\) molecule to be expressed on the cell surface. In addition, we could not detect any mislocalization of the C-terminal deleted \(P_0\) proteins (Y152stop or Q186stop) when coexpressed with wild-type \(P_0\)-EGFP. More recently Xu et al (10) showed that intact cytoplasmic domain of \(P_0\) protein is required for normal adhesive properties of extracellular domain of \(P_0\) molecule in part through the actions of protein kinase C phosphorylation. Based on this study, we would expect that both Y152stop and Q186stop lack the required regions for PKC activation. Whether additional regions of the \(P_0\) intracellular domain are involved in signaling or trafficking remains an open question.

Immunoprecipitation studies suggest that PMP22 dimerizes with itself (31) and can be isolated in complexes with \(P_0\) (7, 29). Previous studies have provided conflicting results regarding the ability of PMP22 mutations to interfere with the trafficking of wild-type PMP22 (18, 19), but this controversy is not directly addressed in this study. We examined whether mutant PMP22 or \(P_0\) proteins could interfere with the trafficking of wild-type \(P_0\) to the cell surface as a basis for the apparent gain of function phenotypes observed in CMT1 and DSS for PMP22 and DSS and CHN for \(P_0\). We found no evidence that any of these mutations interfered with the trafficking of wild-type \(P_0\). Our results are consistent with the hypothesis that self-oligomerization of \(P_0\) and the oligomerization of \(P_0\) and PMP22 probably occurs in a post-ER compartment.

In summary, we have developed PMP22- and \(P_0\)-EGFP fusion proteins as a screening tool for the identification of mutations that interfere with intracellular trafficking of these compact myelin structural proteins. Overall, we find significant differences between the effects of most \(P_0\) mutations and most PMP22 mutations. The majority of PMP22 mutations are associated with abnormal retention of the mutant protein in the ER, whereas the majority of \(P_0\) mutations do not have trafficking defects and are expressed on the cell surface. In doing this, we have confirmed and extended previous studies indicating that the majority of PMP22 mutation result in retention of the abnormal protein in the ER (19). In addition, we have generalized our finding that mutant PMP22 proteins colocalize with calnexin in intracellular structures that are likely associated with degradation of proteins from the ER (21). We have hypothesized that abnormal PMP22-calnexin interactions, either as a consequence of PMP22 overexpression or the presence of a mutation, might underlie the CMT1 neuropathy, and suggested that the formation of abnormal intracellular structures in transfected cells might correlate with this gain of function. We tested whether abrogating the association of mutant PMP22 with calnexin would allow the mutant PMP22 to traverse the ER and access the cell surface as a possible basis for treatment, but we found that the glycosylation-deficient mutant protein was retained in the ER in association with another molecular chaperone, BiP. Despite this result, it is likely that abnormal PMP22/calnexin interactions are important in the pathogenesis of hereditary neuropathies because calnexin null mice have hereditary nerve disease (35) and PMP22 is the major peripheral nerve protein that binds to calnexin (21). We are continuing to study the effects of representative PMP22 “gain of function” mutations, and we have identified 3 new mutations in PMP22 and MPZ in addition to the previously described PMP22 G93R mutation (16) that deserve further study. PMP22V30M appears to be a unique “loss of function” PMP22 missense mutation. \(P_0\)S34del and \(P_0\)G138R will be useful examining the role of \(P_0\) in ER associated protein degradation. We anticipate that additional informative mutations relating to the role of PMP22 and \(P_0\) in
myelin assembly and degradation can be identified using the paradigm described herein.

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