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PMP22 carrying the Trembler or Trembler-J mutation is intracellularly retained in myelinating Schwann cells *in vivo*

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Abstract

The most common cause of human hereditary neuropathies is a duplication in the peripheral myelin protein-22 (PMP22) gene. PMP22 is an integral membrane glycoprotein expressed primarily in the compact myelin of the mammalian peripheral nervous system. The naturally-occurring *Trembler*, and *Trembler-J* mouse models of hereditary neuropathies carry non-conservative point mutations in the PMP22 gene and have been essential in developing the understanding of the cell biology of PMP22 mutants. However, the greatest limitation to the study of PMP22 mutant trafficking has been the lack of a good model of myelination. To address this, we have developed replication-defective recombinant adenoviruses to study the intracellular trafficking of epitope-tagged wild-type, *Trembler*, and *Trembler-J* PMP22 *in vivo*. We have determined that in myelinating Schwann cells *in vivo*, newly synthesized epitope-tagged wild-type PMP22 is incorporated into myelin whereas the PMP22 mutants, *Trembler* and *Trembler-J*, are intracellularly retained in the ER.

Résumé

L'origine la plus commune des neuropathies héréditaires humaines consiste dans la duplication du gène de la protéine de myéline périphérique (PMP22). PMP22 est une protéine intégrale membranaire exprimée principalement dans la myéline compacte du système nerveux périphérique des mammifères. Les modèles murins spontanés de neuropathies héréditaires *Trembler* et *Trembler-J* portent des mutations non-conservatives dans le gène *pmp22* et ont été essentiels dans la progression de nos connaissances sur la biologie cellulaire des mutants de PMP22. L'absence d'un modèle véritable de myélination constitue cependant la limitation principale dans l'étude du transit intracellulaire des mutants PMP22. Pour résoudre ce problème, nous avons construit des adénovirus recombinants permettant d'étudier le transport *in vivo* des differents mutants PMP22 *Trembler* et *Trembler-J*. Nous avons découvert que dans les cellules de Schwann en myélination *in vivo*, PMP22 de type sauvage est incorporé efficacement dans la myéline. Par contre, les mutants PMP22 *Trembler* et *Trembler-J* ne sont pas incorporés dans la myéline, mais sont plutôt retenu dans le réticulum endoplasmique.

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Contribution of Authors

The co-authors appearing on the manuscript contributed to the execution of the experiments, or the preparation of the manuscript in the following manners: Drs. Robert Nicholson and G. Jackson Snipes generated the general purpose cloning vector pBS CMV polio-BP/lacZ. Kathleen Dickson carried out the PMP22 immunoprecipitations. Sandra Aderca provided initial instruction in cell culture techniques. Dr. Wayel Orfali provided intsruction in molecular biological techniques. Drs. Roland Naef and Ueli Suter generated the VSV epitope-tagged wild-type, *Trembler* and *Trembler-J* cDNA. Dr. G. Jackson Snipes conceived of the project, and is the senior author of the manuscript.

The contribution of the author (Joshua Colby) of this thesis in the execution of the experiments and preparation of the manuscript is estimated at 75%.

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1.1 Introduction Charcot-Marie-Tooth disease

In 1886, the French pathologists Charcot and Marie described five patients who suffered from progressive muscular atrophy that started in the feet and legs, and eventually progressed to the hands (Charcot *et al.* 1886). They felt that this often familial affliction had enough stable characteristics to merit its own classification;

"La forme d'atrophie musculaire qui fait l'objet de ce travail nous paraît presenter des caractères assez définis et assez stables pour mériter une description spéciale et une place à part dans les cadres nosographiques."

The same year, Tooth published his Cambridge thesis on a remarkably similar type of progressive muscular atrophy. He concluded that the disease affected the peripheral nerves (Tooth, 1886). Almost a century later, Dyck, Lambert and others would provide neuropathologists with a more concise description of the 'hypertrophic neuropathy of the Charcot-Marie-Tooth type' (Dyck *et al.* 1968): They described CMT as a disorder of dominant inheritance that appeared in the first or second decade of life as foot and gait abnormalities. Predominantly presenting as weakness and atrophy of distal muscles which was most pronounced in the peroneal muscle hence the alternative name 'peroneal muscular atrophy,' CMT would sometimes exhibit moderate distal sensory impairment. As a result of deficient peripheral nerves, nerve conduction velocities were consistently reduced in the ulnar, median and peroneal nerve suggesting demyelination. Histologically, peripheral nerve biopsies revealed axonal and Schwann cell abnormalities. The early collective observations of Charcot, Marie, Tooth, and Dyck are still tenable. As it is known today, Charcot-Marie-Tooth disease (CMT) is one of a heterogeneous group of genetic disorders known as human hereditary motor and sensory neuropathies that lead to progressive degeneration of peripheral nerves. With an estimated incidence of 1:2500, CMT1 is one of the most common genetic disorders known to humankind (Skre *et al.*1974). Therefore, the study of CMT1 disorders is of great relevance in human disease.

With the advent of molecular technology, it has been established that the genes responsible for the most common CMT disorders encode integral membrane glycoproteins expressed by myelinating Schwann cells of the peripheral nervous system. The

identification of the role of these integral membrane glycoproteins in human disease has illustrated their importance in myelin development and maintenance. However, although many of the mutated CMT genes have been identified, the mechanism(s) by which their mutant proteins alter the Schwann cell are still unclear.

Mutations in Peripheral Myelin Protein-22 (PMP22); one of myelin's integral membrane glycoproteins, is the most frequent cause of CMT disease. This thesis will present the design and execution of several experiments that have developed the understanding of the *Trembler* and *Trembler-J* animal models of hereditary neuropathies. These strains of mice have both been described as carrying mutations in the PMP22 gene (Suter *et al.* 1992a,b) The discovery and implication of PMP22 in the pathogenesis of animal and human hereditary neuropathies will be reviewed. To present the rationale for the experiments, a description of the more recent literature will introduce the reader to what is known about the cell biology of PMP22. The experiments are presented in the form of a manuscript. Finally, the potential implications of the experimental findings in terms of understanding of the pathogenic mechanism(s) of PMP22 will be explored.

1.2 Myelin and myelin glycoproteins

Myelinated nerve fibres of the mammalian peripheral nervous system are associated with glial cells that form a specialized insulating membrane structure known as myelin (see Morell, 1984, and references therein). Developmentally, Schwann cells derived from the neural crest follow axonal growth cones into the periphery and proliferate until each large caliber fibre is associated locally with one Schwann cell (Morell, 1984). The promyelin stage is attained when each Schwann cell encloses each axon with one wrap of extended membrane. Following the promyelin stage, each Schwann cell extends this membrane, or *mesaxon*, spirally wrapping an axon 10-100 times. Once wrapped, compaction occurs as the Schwann cell cytoplasm is evacuated bringing the inner and outer membranes into close apposition. This gives rise to a tightly-bound, radially repeating biphasic membrane structure which extends longitudinally along the axon forming an *internode*, defined as the region of the axon bordered by nodes of Ranvier and

myelinated by one Schwann cell (Figure 1). Having a high electrical resistance, myelin acts as an electrical insulator restricting axon depolarization and electrical activity to the nodes of Ranvier. As a result, myelinated fibres exhibit 'saltatory,' or 'jumping' electrical nerve impulse conduction which is much more rapid (20-70m/s) than non-saltatory conduction in nonmyelinated fibres (1m/s). Peripheral myelin is composed of a variety of membrane lipids and glycoproteins. It is rich in cholesterol and long-chain saturated fatty acids. The lipid composition of myelin will not be discussed here. Rather, before focussing on PMP22, a brief introduction to the integral membrane glycoproteins of myelin is warranted as they are the major causative agents in hereditary neuropathies.

Membrane glycoproteins are found within the lipid bilayer of the Schwann cell membrane. Myelin protein zero (P0), a 28 kDa molecule, is the most abundant membrane glycoprotein of peripheral nerve myelin making up at least 50% of peripheral myelin protein (Greenfield *et al.* 1973). Containing an extracellular immunoglobulin-like domain, one membrane spanning domain as well as an intracellular cytoplasmic domain (Lemke *et al.* 1985, 1988), it is thought to provide homophilic adhesion within the intraperiod line (Ding, 1994), and heterophilic adhesion within the major dense line (Filbin *et al.* 1990). To date, at least 57 mutations in P0 have been associated with hereditary neuropathies (reviewed by Nelis *et al.* 1999). Connexin-32 (Cx32) is part of a gene family encoding subunits of gap junctions and is expressed coordinately with other myelin proteins (Bruzzone *et al.* 1997; Scherer *et al.* 1995). Cx32 is present in noncompact regions of peripheral myelin, namely; the paranodes and Schmidt-Lanterman incisures. It is hypothesized that Cx32 oligomerizes to form reflexive intracellular gap junctions which provide cytoplasmic continuity allowing the selective diffusion of small ions and water soluble molecules between adjoining leaflets of the myelinating Schwann cell (Scherer *et al.* 1995, Paul *et al.* 1995). Over 100 mutations in Cx32 have been linked to CMTX, an X-linked form of hereditary neuropathies (Bergoffen *et al.* 1993; Bone *et al.* 1997). Myelin-associated glycoprotein (MAG) exists in two isoforms which are developmentally regulated and contain five glycosylated extracellular immunoglobulin-like domains (Salzer *et al.* 1987; Quarles *et al.* 1973). Localized in the external and periaxonal regions of myelin as well as the Schmidt-Lanterman clefts and the paranodal lateral loops (Martini *et al.*

1994), MAG mutations have not been associated with hereditary neuropathies. The most important myelin integral membrane protein in terms of this discussion is PMP22.

PMP22 has a history that predates its discovery. Indeed, PMP22 has been causing human hereditary neuropathies for over a century and likely much longer. While many questions haunted the study of hereditary neuropathies, this culprit myelin protein was identified only ten years ago. The events that precede and follow the identification of PMP22 form an elegant narrative in which the early unexplained observations of scientists like Charcot, Marie and Tooth have been unified with present molecular and cell biological understanding. Here, we take up the PMP22 narrative with the discovery of a shaky little house mouse in Scotland.

1.3 *Trembler* and *Trembler-J*: Classic animal models of hereditary neuropathies

In 1946 at the Institute of Animal Genetics in Edinburgh, an abnormal strain of mouse that trembled as it walked was discovered. Accordingly, the new strain of mouse was given the name *Trembler*. Five years later, the behavioral characteristics and Mendelian genetics of the *Trembler* mouse were described by Falconer (Falconer, 1951). He reported that in the early stages of life, *Trembler* mice were developmentally retarded, suffered from provoked convulsions and experienced difficulty walking with moderate paralysis of front limbs and pronounced paralysis of the hind limbs. Adult *Tremblers* trembled from side to side while moving; a spastic paresis which subsided when the animal was still. Later in development, *Tremblers* were normal in size, the provoked convulsions subsided and paralysis decreased although it persisted moderately in the hind limbs. Using Mendelian techniques of phenotypic observation in several generations of *Trembler* mice, Falconer observed that the cause of the deficiency was a fully penetrant autosomal dominant gene. Furthermore, considering that the parents of the original *Tremblers* were normal and that the abnormal gene in the heterozygote state was always penetrant with an identifiable phenotype, he concluded that there had been a spontaneous mutation in the 'germplasm' of one of the parents giving rise to the disorder. The appearance of the *Trembler* shaky house mouse gave birth to a new field of study.

Researchers immediately looked for disorders in the central nervous system of *Trembler* mice hoping to find an animal model of central nervous system disorders. These early histological and functional examinations failed to reveal any abnormalities (Braverman *et al.* 1952). Unfortunately, they overlooked the peripheral nervous system.

It was not until 1973 that the first complete histological analysis of the peripheral nervous system revealed that *Trembler* mice suffered from severe peripheral nerve deficits (Ayers *et al.* 1973). Examination of the sciatic nerve from 14-day-old *Tremblers* revealed delayed onset of myelination, Schwann cells with enlarged cytoplasm containing myelin debris, hypertrophied perineurium, axons with several loops of uncompacted myelin, and segmental demyelination. In adult *Tremblers*, most sciatic nerve fibres were hypomyelinated and contained an increased number of Schwann cell nuclei as well as onion bulbs; poorly myelinated axons with redundant loops surrounded by excessive basal lamina. In addition, the Schwann cell Golgi apparatus, endoplasmic reticulum, and mitochondria were hypertrophied. Myelin debris was observed in the cytoplasm. Thus, it appeared that younger animals displayed delayed onset of myelination, while older animals displayed hypomyelination, increased glial cell number, and evidence for cycles of demyelination and remyelination.

A functional assessment of peripheral-nerve function in the *Trembler* mouse revealed physiological deficits characteristic of demyelinated nerves (Low *et al.* 1975). These deficits were consistent with the observed *Trembler* neuropathies. Compound muscle action potential (CMAP) amplitude, terminal latency and motor conduction velocities were abnormal. The CMAP of *Trembler* mice was reduced to 0.21 ± 0.13 mV compared to that of the wild-type; 3.18 ± 1.15mV. The distal latency of the sciatic nerve was increased 4.16 ± 2.01 msec relative to wild type; 0.92 ± 0.21 msec. The motor conduction velocity was greatly decreased; 45.92 ± 7.21 m/sec in wild-type and 2.51 ± 1.34 m/sec in *Trembler* mice. Relative to wild type mice, *Trembler* mice did not develop increasing NCV with age. In addition, histological analysis of these nerves uncovered axonal abnormalities. *Trembler* mice had decreased nerve fibre density; 6.43×10^3

fibres/mm^2 compared to the normal which was 17.92 x 10^3. Large diameter fibres were most severely affected in *Tremblers*. While fibres of 7-9 μm diameter could be found in normal adult mice, few nerve fibres over 6 μm could be found in the *Tremblers*. This was one of the first indications of deficient axon-glial interaction in the *Trembler* mouse. Soon thereafter, Ayers *et al.* 1976) carried out an extensive quantitative electron microscope morphometric study of trember animals at various ages during development and adulthood and found that relative to normal mice, the mean axon diameter of *Trembler* nerves was decreased at all ages. There was delayed myelination, with abnormally thin myelin when present, and myelin thickness relative to axon diameter was decreased at all ages.

At this time, it was not clear whether the *Trembler* mutation manifested in the axon or in the Schwann cell as both presented visible abnormalities. In 1977, Perkins *et al* carried out a study that addressed this question. Having previously established that if a section of sciatic nerve was explanted from one mouse into another, the Schwann cells from the donor nerve would ensheath the regenerating axons of the recipient sciatic nerve (Aguayo *et al.* 1976), they carried out nerve transplants between normal and *Trembler* mice. They found that normal nerves explanted into normal nerves developed normal morphology. Normal nerves explanted into *Trembler* nerves also developed normal morphology. In contrast, *Trembler* nerves explanted into normal nerves assumed the morphological characteristics of a *Trembler* nerve, namely; hypomyelination and absence of myelin. This experiment indicated that the *Trembler* mutation resulted in a primary disorder of *non-neuronal* cells, not the axons.

Observing an increased number of Schwann cells, Perkins *et al.* (1981) further studied Schwann cell proliferation in *Trembler* nerves. Relative to normal nerves, *Trembler* sciatic nerves had two-fold the number of Schwann cell nuclei at P10, five-fold the number at 1 month and ten-fold the number at 4 months. Using grafting experiments similar to those above, they determined that the excessive proliferation of Schwann cells was also inherent to the mutant Schwann cells. Noting that the Schwann cells would frequently ensheath the axon but fail to myelinate, they concluded, '*the Trembler mutation curtails differentiation beyond the stage of primary ensheathment.*'

Given the peripheral nerve deficits of *Trembler* mice, it was not surprising that the neuromuscular junctions were affected. Voluntary muscles contained atrophied fibres that were hypothesized to have resulted from partial denervation (Koenig *et al.* 1980). Consistent with this idea, indications of endplate degeneration and regeneration were also reported, namely; abnormal axonal branching, swelling on terminal branches, terminal and preterminal sprouting, multinervated muscle fibers as well as axons innervating more than one fiber.

In 1979, thirty years after the first *Trembler* was detected, a second strain of trembling mice appeared in the Jackson laboratories, Maine (Sidman *et al.* 1979). Named the *Trembler-J* mouse, it carried a mutation co-isogenic with C57BL/6J mice and was unrelated to the *Trembler* mouse. Relative to *Tremblers*, *Trembler-J* mice had a smaller amplitude tremor with no apparent seizures, and a milder gait abnormality that did not deteriorate with age (Henry *et al.* 1983a,b). In contrast to heterozygote *Trembler* mice which could be phenotypically identified by P20, the *Trembler-J* heterozygote mice were not clearly identifiable until P25 as a result of their more moderate phenotype. *Trembler-J* heterozygote mice had a more moderate myelin deficiency relative to *Trembler* mice: Most large fibres had at least thin myelin sheaths; a pattern which persisted in older animals with appropriately sized fibres having at least a thin myelin sheath. No onion bulbs were present in *Trembler-J* mice. *Trembler-J* sciatic nerves had abnormally high numbers of Schwann cell nuclei, increased fibroblasts and increased interstitial connective tissue although these were not as marked as in *Tremblers*. The *Trembler-J* homozygote mice showed a severely dystonic phenotype by P10 and did not survive long enough to wean Homozygote *Trembler-J* sciatic nerve was characterized by a severe myelin deficit with few if any myelinated fibres present. Consistent with this, the *Trembler-J* homozygote mouse is thought to be the most lethal hereditary neuropathy documented (Henry *et al.* 1983).

It had been previously established that the *Trembler* locus was closely linked to the vestigial tail gene (vg) on chromosome 11 (Davisson *et al.* 1981). Using this knowledge in backcrossing experiments, Henry *et al.* (1983) discovered that the recombination frequency

between *Tr* with *vg*, and *Tr-J* with *vg* was almost identical. This provided the first genetic indication that the *Trembler* and *Trembler-J* mutations might occur within the same gene

In producing two strains of mouse hereditary neuropathies, nature supplied science with an opportunity to study peripheral myelin in both health and disease. A new branch of neuroscience was created and devoted to studying the *Trembler* and *Trembler-J* neuropathies. Between 1980 and 1990, a plethora of studies were carried out in the hopes of uncovering the Schwann cell deficit in *Trembler* and *Trembler-J* mice. It became apparent that the Schwann cells from these mice were abnormal in many ways; cell cycle, lipid and protein synthesis, as well as structural integrity (Henry *et al.* 1983; Bourre *et al.* 1984). However, the primary defect of these Schwann cells was not uncovered until 1992.

1.4 Discovering PMP22; the *Trembler* and *Trembler-J* protein

In the late eighties, a gene that appeared to be upregulated during growth arrest in mammalian cells was identified in NIH 3T3 cells (Schneider *et al.* 1988). It was given the name *g*rowth *a*rrest *s*pecific gene-3 (*gas-3*). Manfioletti *et al.* (1990) characterized the expression of this novel cDNA that appeared to be influenced by the state of the cell cycle. They determined that *gas-3* mRNA disappeared within 6 hrs of serum induction of growth arrested NIH 3T3 cells. Conversely, *gas-3* mRNA reappeared within 12 hrs in newly serum starved NIH 3T3 cells. During density-dependent growth inhibition *gas-3* mRNA increased between 2-6 days. The full *gas-3* cDNA sequence was 1,817 nucleotides predicting a 144 amino acid chain with one site for N-linked glycosylation and three potential membrane spanning domains. *In vitro* translation assays determined that the protein had a molecular weight of 18 kDa unglycosylated, and 22 kDa under glycosylating conditions. Phase separation experiments showed that *gas-3* was an integral membrane protein.

In 1991, Welcher *et al.* (1991) carried out differential screening of a cDNA library constructed from crushed versus non-crushed rat sciatic nerve. They isolated and characterized one clone of which the mRNA was expressed in sciatic nerve and appeared

to be downregulated 1 day after nerve crush and subsequently upregulated from 3-14 days. Sequencing of this clone, SR13, revealed a reading frame of 460 nucleotides predicting a 160 amino acid chain with a molecular weight of 18 kDa containing four potential membrane spanning domains and one site for N-linked glycosylation. They reported that this new clone shared 98% homology with *gas-3*. Using a polyclonal antibody against SR13, they immunolocalized SR13 to myelin and proposed that it was a myelin protein.

The following year, Snipes *et al.* (1992) further characterized the anatomical localization of SR13 as well as its expression during myelin development and following nerve crush. A thorough immunohistochemical study of SR13 revealed that it was expressed primarily in the myelin sheath of the peripheral nervous system. Western blot analysis of whole protein from sciatic nerve demonstrated that the SR13 antisera recognized a 22 kDa protein. They named the protein Peripheral Myelin Protein-22 and proposed that *gas-3*, SR13 and PMP22 were the same protein after correcting a sequencing error in the original *gas-3* sequence. In the developing rat, mRNA and protein expression of PMP22 correlated with myelin development with low expression (10% maximum) shortly after birth, and induced expression reaching a maximum three weeks after birth. PMP22 mRNA and protein expression was also temporally regulated following sciatic nerve crush injury. In situ mRNA hybridization of peripheral nerves revealed that Schwann cells were indeed generating the PMP22.

Having identified, characterized and named PMP22, the same team discovered that a mutation in the PMP22 gene existed in the *Trembler* mouse (Suter *et al.* 1992). Using rat-mouse somatic cell hybrids with PCR primers specific for PMP22 (*gas-3*), they localized PMP22 to chromosome 11. Cloned and sequenced PCR products from *Trembler* sciatic nerve RNA revealed two alleles; one wild-type and one variant which contained a guanine replaced by an adenine in the coding sequence of PMP22. The variant allele in the *Trembler* mouse contained a non-conservative point mutation that introduced an aspartic acid for a glycine residue at position 150 in the amino acid sequence (G150D). Effectively, the mutation introduced a charged amino-acid into the fourth putative membrane spanning

domain of the protein. This was the first indication that PMP22 is a disease gene in hereditary neuropathies.

One year later, using interspecific backcross experiments with *Trembler* mice, Suter *et al.* determined that the location of PMP-22 on mouse chromosome 11 lay between two previously characterized chromosome loci on chromosome 11 (Buchberg *et al.* 1989; Suter *et al.* 1992). One of these loci was syntenicto a region on the short arm of human chromosome 17. Furthermore, when they extracted total RNA from *Trembler-J* mouse sciatic nerves, reverse-transcribed, subcloned and sequenced it, they discovered a non-conservative thymidine to cytosine substitution which would introduce a leucine to proline substitution in the first putative membrane spanning domain of the PMP22 amino acid sequence. The isolation of a second PMP22 point mutation in a mouse model of hereditary neuropathies clearly indicated that PMP22 could cause hereditary neuropathies.

Forty years after the first trembling mouse had been identified, it became clear that the fundamental Schwann cell deficiency was a mutated myelin protein; PMP22. Understandably, PMP22 became a candidate gene in the development of human hereditary neuropathies.

1.5 Implicating PMP22 in CMT disease

While PMP22 was being discovered and pursued in mice, researchers were attempting to localize the gene(s) responsible for Charcot-Marie-Tooth type 1 (CMT1a); the most common form of human hereditary neuropathy (Skre et al. 1974). In 1989, Vance *et al.* combined genotyping in parallel with neurophysiological assessments on six families with CMT1a and found the disorder in these families was linked to markers on chromosome 17. Two-point and multipoint linkage tests of a multi-generation family with CMT1a further indicated that the CMT1a mutation was located on the small arm of chromosome 17 (Faeymaekers *et al.* 1989). Soon thereafter, Lupski *et al* (1991) utilized a number of molecular and genetic techniques to identify a local DNA duplication on chromosome 17p in seven mutligenerational pedigrees afflicted with CMT1a. Within this

cohort, they reported one severely affected individual who was homozygous for the duplication (4 copies). This was the first indication that overexpression of a duplicated gene could be the primary problem in CMT1a. The gene dosage theory was corroborated when patients who suffered from electrophysiological deficits characteristic of CMT1a were reported as having a 17p deletion (Chance *et al.* 1992). Chance *et al.* further proposed gene dosage as the fundamental problem in CMT.

It was in 1992 that PMP22 was discovered on mouse chromosome 11 and identified as the pathogenic gene encoding a myelin gene which caused the neuropathies in the *Trembler* and *Trembler-J* mouse models of hereditary neuropathies. As mouse chromosome 11 and human chromosome 17 contained regions of conserved synteny (Buchberg *et al.*1989), PMP22 became a prime suspect in the search for the CMT1 gene.

Patel *et al.* (1992) were the first to clone and sequence human PMP22 that mapped to the duplicated region on chromsome 17 and encoded a 160 amino acid protein that shared 87% homology with rat PMP22 and 86% homology with mouse. They reported that, like the mouse counterpart, human PMP22 mRNA was expressed primarily in peripheral nerve and spinal cord. Simultaneously, a number of groups localized PMP22 within the CMT1a duplicated region on chromosome 17p11.2-12 (Valentijn *et al.* 1992; Timmerman *et al.* 1992; Matsunami *et al.* 1992). Interestingly, three pedigrees with individuals suffering from hereditary neuropathy with liability to pressure palsy (HNPP) were found to be missing markers for the CMT1a duplication region including PMP22 (Chance, 1993). HNPP is an autosomal dominant form of hereditary neuropathy characterized by recurrent sensory and motor dysfunction that is often triggered by minor trauma (Behse *et al.* 1972). Studying these individuals, Chance *et al.* (1993) located a 1.5 Mb deletion on chromosome 17p11.2-12, and proposed that it was the reciprocal deletion to the CMT1a duplication. A clinical and electrophysiological study of 119 genetically homogeneous CMT patients with the 17p11.2 duplication showed that motor nerve conduction velocity changes segregated with the duplication (Birouk *et al.* 1997).

The connection between the *Trembler-J* mouse model and CMT became irrefutable when Valentijn *et al.* (1992) described a CMT family that carried the *Trembler-J* thymidine to cytosine mutation in the PMP22 coding region (L16P) and in which the affected individuals suffered from clinical and neurophysiological signs of CMT. They further linked this mutation to the disease in 10 family members and reported complete segregation of the PMP22 point mutation with CMT1a in the family. Much later, Ionasescu *et al.* (1997) would report a mother and son who suffered from Dejerine-Sottas-Syndrome, another form of human hereditary neuropathy, and who carried the *Trembler* glycine to aspartic acid (G150D) substitution in their coding region. These two findings have emphasized the intimacy of the *Trembler* and *Trembler-J* mutations with human hereditary neuropathies.

It is now unambiguously established that the PMP22 duplication plays a causative role in the majority of human hereditary neuropathies. Furthermore, 27 distinct mutations in the PMP22 gene have been identified in different individuals suffering from various more rare forms of hereditary neuropathies (reviewed by Nelis *et al.* 1999; and Gabreel-Festen *et al*, 1999). The discussion will now turn to what is currently known about PMP22 to introduce the rationale and design of experiments presented in the manuscript.

1.6 Studying PMP22

1.6.1. PMP22 as it is known today

PMP22 is a 160 amino acid, 22 kDa integral membrane glycoprotein with four putative membrane spanning domains and two extracellular loops (Figure 2, Welcher *et al.* 1991, Spreyer *et al.* 1991). The first extracellular loop is post-translationally glycosylated and contains the L2/HNK1 epitope which is known to be involved in adhesive interactions (Snipes *et al.* 1993, Griffith *et al.* 1992). Both termini of PMP22 are exposed to the cytoplasm (D'Urso *et al.* 1997). PMP22 is expressed under the control of two alternative putative promoters giving rise to transcripts containing one of two untranslated exons, 1A or 1B, 5'-prime to the translated sequence (Suter *et al.* 1994). Expression of the alternative

transcripts occurs in a tissue- and cell-specific manner. The 1A containing transcript is relatively specific to myelinating Schwann cells and is upregulated 25-fold during postnatal myelin development and during myelination following nerve crush injury. In contrast, the 1B containing transcript is not regulated preferentially and is found predominantly in non-neural tissue. In this manner, PMP22 is expressed primarily in myelinating Schwann cells of the PNS where it comprises 5-10% of total myelin protein and is incorporated into compact myelin (Snipes *et al.* 1992, Kuhn *et al.* 1993, Pareek *et al.* 1993). PMP22 mRNA and protein have been reported in motoneurons, while transcripts have been reported in brain, lung, intestine, and heart (Patel *et al.* 1992, Manfioletti *et al.* 1990, Suter *et al.* 1994, Parmantier *et al.* 1995).

Recently, PMP22 has been identified as a member of a novel gene family with epithelial membrane protein-1 (EMP-1), EMP-2, EMP-3 and major lens fiber protein MP20 (Taylor *et al.* 1995). Sharing some homology, these proteins demonstrate epithelial-specific expression and have been hypothesized to play multiple roles in cell proliferation and differentiation. PMP22 might be related to another novel and rapidly growing gene family comprising the claudin membrane tetraspan proteins (Morita *et al.* 1999). The claudins appear to play an important role in the formation of diverse endothelial and epithelial tight junctions (Morita *et al.* 1999). Furthermore, oligodendrocyte-specific-protein(OSP)/claudin-11 is essential in the formation of parallel array tight junctions found in CNS myelin (Gow *et al.* 1999). Clarification of the similarities, if any, between PMP22 and the claudins might provide insights into the function of PMP22 which at this point is unclear.

1.6.2. PMP22 Knockouts and Overexpressors

Creating animals lacking a newly identified gene is a common method of exploring the essential function(s) of the encoded protein. PMP22 knockout mice have revealed that the proper level of PMP22 expression, corresponding to two wild-type PMP22 alleles, is essential to establishing and maintaining myelin thickness and stability (Adlkofer *et al.* 1995). Behaviorally, homozygote knock-outs display stress-induced tremors and

experience difficulty walking with mild tremor and progressive paralysis of the hind-limbs. Homozygote knock-out mice demonstrate electrophysiological disorders characteristic of peripheral myelin disorders, namely, slowed motor nerve conduction, dispersion and decreased amplitude of compound muscle action potential, as well as increased motor latencies. Morphological analysis of these mice at various ages reveal myelin and axon disorders. At P24, there are prominent myelin thickenings consisting of redundant myelin loops (tomacula) in which the corresponding axons appear compressed. Large caliber fibres lacking myelin can also be found. The myelin tomacula disappear by 10 weeks at which point signs of myelin degeneration, namely thinly myelinated axons with onion bulbs and excessive basal lamina, can be detected. Consistent with a decrease in large caliber axons, animals present muscle pathology as indicated by muscle fibre type grouping of the quadriceps muscle. This is hypothesized to be consistent with acute denervation and nerve sprouting (3 month). During myelin development, it appears the knock-outs form the promyelin Schwann cell to axon 1:1 relationship, but myelination is delayed relative to the wild-type. Interestingly, the heterozygote knock-out is indistinguishable from the wild-type in terms of behavior with fairly normal motor and mixed afferent conduction at 10 weeks of age. The heterozygote morphological phenotype is also more moderate with rare tomacula, no apparent axonal loss, and some tomacula at 10 weeks with occasional onion bulbs. A longer time-course analysis of heterozygote mice reveals that at 12-14 months of age, the only electrophysiological deficit is reduced M-response amplitudes (Adlkofer *et al.* 1997). Between 5-10 months, there is an increase in myelin thickness relative to axon caliber. By 15 months, the morphology is characterized by persistent hypermyelination, often in the form of tomacula, but additional appearance of hypomyelinated nerves and onion bulbs.

Reciprocally, mice were generated that carry 16 copies of the PMP22 gene leading to a two-fold increase in PMP22 mRNA expression relative to P0 mRNA expression (Magyar *et al.* 1996). In these mice, both PMP22 expression and P0 expression are reduced relative to that of normal mice as a result of the altered Schwann cell biology of the overexpressors. Overexpressors shiver at two weeks, develop an unsteady gate with signs of muscle atrophy at four weeks, and finally a progressive paralysis of the hindlimbs.

Typical signs of demyelinating neuropathy, although variable, are noted. These include increased latencies and compound muscle action potential dispersion with decreased amplitude. In early myelin development (P10), Schwann cells develop to the 1:1 Schwann cell to axon ratio with a basal lamina. However, myelination fails to commence. The Schwann cell cytoplasm grows more electron dense and there is excessive deposition of basal laminae. As in the knock-outs, there is muscle fibre type grouping, with neurogenic atrophy, although no signs of nerve fibre degeneration are observed. Corroborating these findings, a number of transgenic mouse lines with 40 kb human PMP22 gene have been generated (Huxley *et al.* 1998). In these lines, demyelination appears and worsens when the ratio of human to mouse mRNA in transgenic lines approaches and exceeds 1.0, respectively. The highest expressers have little or no myelin in the periphery. Electrophysiologically, motor nerve conduction velocities are decreased and distal motor latencies increased in a similar pattern. Electron microscopic examination reveals uncompacted myelin, demyelinated large axons, and duplicated basal laminae. In sum, the Schwann cells of mice overexpressing PMP22 appear to be halted at the promyelin state.

The observation that the heterozygous *Trembler* and heterozygous PMP22 knockout mice have different phenotypes lead to the hypothesis that the *Trembler* allele have a 'gain-of-function' effect on Schwann cells. To address this issue, Adlkofer *et al.* mated *Trembler* and knockout mice and compared the effect that the *Trembler* allele produced with, or in the absence of, the wild-type allele (Adlkofer *et al.* 1997). Indeed, the mere presence of the *Trembler* allele consistently results in dramatic hypomyelination in (*Tr*/+), (*Tr*/0), and (*Tr*/*Tr*) mice while age-matched homozygote (0/0) and heterozygote (0/+) knock-outs display only focal hypermyelination with no signs of hypomyelination. This has verified that the *Trembler* allele has a dynamic effect on Schwann cells. Furthermore, the drastic difference between the *Trembler-J* homozygote animals and the homozygote knock-outs implies that the *Trembler-J* allele also exerts an dynamic effect on myelinating Schwann cells (Henry *et al.* 1983a,b, Adlkofer *et al.* 1995). The effect of the *Trembler* and *Trembler-J* mutants on the Schwann cell has sparked much interest in the cell biology of PMP22.

1.6.3. The Cell Biology of PMP22

Pareek *et al.* (1993) studied the cell biology of PMP22 in forskolin-induced myelinating Schwann cells *in vitro*. Schwann cells under these conditions express a 1.8 kb RNA transcript encoding PMP22. However, less than 5% the amount of PMP22 is produced relative to the amount produced in neonatal sciatic nerve Schwann cells reflecting the limitations of the *in vitro* system. Both *in vitro* and *in vivo*, an antiserum raised against PMP22 immunoprecipitates a 22 kDa protein and to a lesser extent a 18 kDa protein. Application of tunicamycin (which inhibits N-linked glycosylation), *in vitro* and *in vivo* reveals that the 18 kDa band is a transient product which is post-translationally glycosylated giving rise to the 22 kDa mature protein. Immunohistochemical analysis of Schwann cells *in vitro* show PMP22 diffuse throughout the cytoplasm but surprisingly not on the cell membrane. Pareek *et al.* also report that mRNA levels in the adult nerve are decreased relative to the amount of protein. This suggests that once made, PMP22 protein is fairly stable in myelin.

The cellular localization and membrane topology of PMP22 expressed in HeLa cells has been described by D'Urso *et al.* (D'Urso *et al.* 1997). PMP22 localizes to the region of the endoplasmic reticulum, the Golgi compartment, and on the cellular membrane. The amino and carboxy terminus are cytoplasmic while two hydrophilic sequences form extracellular loops containing amino acids 28-40, 118-131, including Asp-41, the concensus site for glycosylation.

The discovery of PMP22 as homologous to *gas-3* opened the possibility that PMP22 might act as a regulator of the Schwann cell cycle. Furthermore, given that PMP22 is upregulated when myelination begins, that it is regulated in a cell cycle specific manner in NIH 3T3 cells, and that the mouse mutants *Trembler* and *Trembler-J* are marked by Schwann cell hyperproliferation, it is often theorized that PMP22 influences the Schwann cell cycle. Some studies have explored this possibility. In various stages of the Schwann cell cycle, PMP22 mRNA levels follow the same pattern as those seen in NIH 3T3 cells during quiescence and entry into synthesis phase (Zoidl *et al.* 1992, Manfioletti *et al.*

1990). A retroviral overexpression of PMP22 in cultured Schwann cells suggests that overexpressors exhibit moderately decreased proliferation as well as slightly delayed entry into the synthesis phase of the cell cycle following mitogen stimulation. Fabretti *et al.* report that in NIH 3T3 cells, overexpression of PMP22 leads to an apoptotic-like phenotype with rounding and blebbing of cells although no DNA fragmentation is evident (Fabretti *et al.* 1995). A similar retroviral approach in myelinating co-cultures suggests that overexpression of PMP22 has a similar effect on the proliferation of Schwann cells (D'Urso *et al.* 1997). With the exception of excessive PMP22 in the perinuclear region, overexpression does not affect myelin development *in vitro*. Reciprocal underexpression has little or no effect on myelination *in vitro*. As of yet, there is no direct evidence for a mechanistic pathway by which PMP22 can alter the Schwann cell cycle.

Recently, there has been a focus on improving the understanding of the cell biology of PMP22 in the hopes of uncovering discrepancies between the wild-type and the mutants. Pareek *et al.* (1997) carried out *in vitro* and *ex vivo* metabolic labeling experiments in combination with EndoH assays to determine the time-course of PMP22 synthesis in Schwann cells. Newly synthesized PMP22 in cultured Schwann cells is mostly EndoH sensitive indicating that it is in a pre-Golgi compartment. This pool of EndoH sensitive PMP22 is degraded within 30-60 minutes. A small pool of newly synthesized PMP22 is EndoH resistant and is localized to the Golgi compartment with none detectable on the cell membrane. Identical biochemical experiments on myelinating Schwann cell/axon co-cultures as well as *ex vivo* labeled 10-day-old rat sciatic nerve similarly show a large pool of EndoH resistant PMP22 with a rapid turnover. Repeated in older animals and co-cultures in which myelin is fully developed, this experiment reveals a small pool of EndoH sensitive PMP22 as well as a larger pool of EndoH resistant PMP22 which likely comes from the Golgi compartment and myelin membrane. Pareek *et al.* observed that translocation of PMP22 from the Schwann cell Golgi to the membrane was increased when axons were present. This lead them to conclude that axonal contact promotes the translocation of PMP22 from the Golgi into myelin. This emphasizes the influence of axonal contact on Schwann cell biology. In sum, persistent fully glycosylated PMP22 in

Schwann cells comes from the processing and accumulation of a small portion of the total PMP22 produced.

Initial observations of an *in vivo* study of P18 heterozygote *Trembler-J* mice indicated that PMP22 protein levels are reduced disproportionately to P0 and MBP (Notterpek *et al.* 1997). Endo H sensitive PMP22 in these animals is not increased indicating that ER retention is less likely although compensation by the normal allele cannot be ruled out. Accumulation of PMP22 in the perinuclear region of SC is observed in transverse sections of sciatic nerve which overlap partially with endosomal markers LAMP-1, but not with ER markers. Further examination of the endosomal-lysosomal pathway reveals an upregulation of LAMP-1, a structural component of lysosomes (Chen *et al.* 1985), and cathepsin D, a marker for lysosomal enzyme activity (Cataldo *et al.* 1995), in heterozygote *Trembler-J* sciatic nerve relative to the wild-type. When lysosomal activity is blocked with chloroquine treatment, a greater accumulation of PMP22, P0 and MBP is observed in heterozygote *Trembler-J* relative to wild-type nerves. Based on these observations, a mechanism of increased endocytosis and autophagy has been proposed as a component of the pathogenic pathway of *Trembler-J* PMP22.

Much of the current literature supports a theory of aberrant intracellular trafficking of PMP22 mutants. Naef *et al.* (1997) have reported increased PMP22 immunostaining in the perinuclear region of hypomyelinating Schwann cells in *Trembler* heterozygote mice. Consistent with this observation, when wild-type and *Trembler* PMP22 are transiently transfected into COS-7 cells, the *Trembler* PMP22 is retained in a bloated endoplasmic reticulum whereas the wild type is transported successfully through the ER and Golgi apparatus to the membrane. In addition, co-transfected *Trembler* PMP22 may exert a dominant negative effect on the transport of wild-type PMP22 to the membrane of COS-7 cells. In hemizygous animals (0/*Tr*), residual myelin stains positively for P0 but not for PMP22, although it must be noted that no PMP22 can be detected at this age. Naef *et al* have proposed ER retention as a disease mechanism.

Other *in vitro* studies on the intracellular trafficking of the *Trembler* and *Trembler-J* mutants support this hypothesis (D'Urso *et al.* 1998). In HeLa cells and primary Schwann cell cultures, wild-type PMP22 is localized in the ER, the Golgi apparatus, and on the membrane with a small amount colocalizing with lysosomal markers. In contrast, the *Trembler* and *Trembler-J* PMP22 remain localized to the ER, with a small amount colocalizing with lysozomal markers. D'Urso *et al.* observe no dominant negative effect of the mutants on wild-type trafficking. The Schwann cells of a nerve from an afflicted CMT1a, analyzed immunohistochemically, also reveal cytoplasm overloaded with PMP22. These observations indicate that the *Trembler* and *Trembler-J* mutations might cause abnormal intracellular processing of PMP22.

An *in vitro* study has introduced another intracellular organelle into the retention theory (Tobler *et al.* 1999). Whereas wild-type epitope-tagged PMP22 transiently transfected in COS-7 cells is rendered EndoH resistant and effectively transported to the membrane, *Trembler-J* PMP22 remains EndoH sensitive, alters the morphology of the ER and can be visualized in large vesicular-like structures which colocalize with ERGIC53, a marker for the intermediate compartment. When wild-type and *Trembler-J* PMP22 are co-transfected, the wild-type assumes the ERGIC-like distribution characteristic of *Trembler-J*. Supporting this, it has been determined biochemically that *in vitro*, wild-type and *Trembler-J* can form heterodimers, a process which seems to be independent of the glycosylation state of the molecules. Tobler *et al.* project that the *Trembler-J* is primarily stuck in the ERGIC compartment. Taken together, these studies suggest that aberrant intracellular trafficking could be a common disease mechanism of PMP22 mutations.

There is good indication that newly synthesized PMP22 is degraded through a cytoplasmic degradation pathway involving the proteasome (Notterpek *et al.* 1999). The ubiquitin-proteasome pathway is an elaborate, regulated pathway of intracellular degradation (reviewed by Ciechanover. 1994) that has recently been identified as a component of ER-associated degradation of the cystic fibrosis transmembrane conductance receptor (CFTR, Ward *et al.* 1995). Selective inhibition of the proteasome pathway in cultured cells expressing CFTR *in vitro* leads to the formation of cytoplasmic inclusions of

polyubiquitinated CFTR (Doan *et al.* 1996). These intracellular inclusions have been named aggresomes and are thought to constitute a cellular response to saturation of the ubiquitin-proteasome degradation pathway (Johnston *et al.* 1998) Notterpek *et al* have found that inhibition of the proteasome pathway in cultured cells expressing PMP22 similarly leads to accumulation of PMP22 in aggresomes (Notterpek *et al.* 1999). This suggests that like the CFTR, PMP22 might be degraded via the cytoplasmic proteasome pathway.

To summarize, of a large pool of PMP22 translated into the ER, a small amount is transported through the Golgi compartment where complex-glycosylation occurs, and finally transported to the membrane. Newly synthesized PMP22 might be degraded through the cyoplasmic proteasome machinery. In addition to a potential increase in degradation of the mutants via the endosomal-lysosomal pathway, the above studies support the idea that the *Trembler* and *Trembler-J* forms of PMP22 are trafficked through mammalian cells in a different manner than the wild-type. However, the conclusiveness of the observations on the intracellular processing of PMP22 are limited for one simple reason: the degree to which cultured cells represent myelinating Schwann cells *in vivo* is not established.

1.7. Rationale for an *in vivo* approach to studying the intracellular processing of PMP22 mutants

The interdependence of Schwann cells and axons is well established. Early experiments showed that sensory axons are mitogenic to Schwann cells, axonal transection leads to Schwann cell death, and the presence of axons helps to determine glial numbers (Wood *et al.* 1975, Aguayo *et al.* 1976, 1977, 1981). Despite a lack of knowledge of the mechanisms by which Schwann cells and axons interact, local modulatory factors and Schwann cell transcription factors are slowly being characterized. Some examples are summarized below and are illustrative of the importance of axon-glial interaction to the Schwann cell.

Reporting axon-glial interaction, Raff *et al.* (1978) observed an unidentified secreted protein that acted as a glial cell mitogen and seemed to play several roles in the developing Schwann cell. Now characterized, Neuregulin-1 (NRG-1) is expressed by neurons and influences Schwann cell precursor proliferation and differentiation during prenatal development and after nerve injury (Murphy *et al.* 1996, Meyer *et al.* 1995, Chen *et al.* 1997). Axonally derived NRG1 acts as a Schwann cell survival factor early postnatally (Grinspan *et al.* 1996). NRG1 is downregulated after myelination at which point its influence on Schwann cells is unclear. A number of transcription factors are differentially active in the Schwann cell. Pax3 is a homodomain protein important in Schwann cell development and may play a role in determining myelinating versus non-myelinating phenotype (Kioussi *et al.* 1995). Suppressed cAMP inducible POU protein (SCIP/TST1/Oct6) is a transcription factor whose expression is dependant on axonal contact (Aroyo *et al.* 1998). Expression of SCIP/Tst1/Oct6 is high as the Schwann cells enter the promyelinating state and they decline as myelination commences (Blanchard *et al.* 1996). Krox-20/Erg2 is a zinc finger protein which is expressed in the PNS with the appearance of embryonic Schwann cells (Topilko *et al.* 1994) whose expression is dependant on axonal contact and is upregulated following induction of SCIP/TST1/Oct6 (Zorick *et al.* 1996). Although brief, these examples illustrate that the Schwann cell can be influenced by local trophic factors and by axonal contact. Furthermore, Schwann cells seem to be controlled by the collective action of many transcription factors (reviewed by Wegner *et al.* 2000).

Local environmental factors could have a profound effect on the cell biology of the Schwann cell. In support of this, *in vivo* Schwann cells producing myelin early in development are capable of handling a two hundred-fold increase in the expression of PMP22 concomitant to induced transcription (Snipes *et al.* 1992, Suter *et al.* 1994). In contrast, Schwann cells co-cultured with axons and provoked into a myelinating state produce less than 5% the amount of PMP22 produced by myelinating Schwann cells *in vivo* (Pareek *et al.* 1993). Such a marked discrepancy between the *in vitro* and *in vivo* production of PMP22 could result from differences in the local environment. Based on this, it is possible that a Schwann cell is best equipped to process PMP22 in its natural

environment where the influence of axon-glial signals and local trophic factors are present. Similarly, a myelinating Schwann cell could be capable of processing mutant forms of PMP22 to a greater degree than cells grown *in vitro*. Therefore, to study the intracellular trafficking of PMP22, it seems that an *in vivo* approach is appropriate.

1.8. Setting up a system to study the intracellular trafficking PMP22 *in vivo*

The fundamental aim of this project was to study the intracellular trafficking of the *Trembler* and *Trembler-J* mutants in a myelinating Schwann cell *in vivo*. To devise an effective system to do this, three requirements had to be addressed: First, a means by which myelinating Schwann cells could be transduced *in vivo* was required. Second, the expressed *de novo* PMP22 had to be visualized against endogenous PMP22. Finally, an effective means of assessing Schwann cell transduction was required.

To express the various forms of PMP22 *in vivo*, a recombinant adenoviral approach was utilized. Adenoviruses are capable of infecting non-dividing mammalian cells such as myelinating Schwann cells. Moreover, the molecular techniques are available to introduce expression cassettes into the genomic backbone of adenoviruses that have been rendered replication-defective. Replication-defective recombinant adenovirus can effectively transduce Schwann cells without triggering the adenoviral replication cycle within the Schwann cell. To visualize newly synthesized adenoviral-derived PMP22, a VSV-G protein epitope tag expressed on the carboxy terminus of PMP22 was used. In this way, adenoviral derived PMP22 could be visualized using a polyclonal antibody generated against the VSV epitope tag. Finally, the *lacZ* reporter gene was introduced into the expression cassette providing an effective means of determining transduction efficiency.

After generating replication-defective adenoviruses expressing epitope-tagged wild-type, *Trembler* or *Trembler-J* PMP22 as well as the *lacZ* reporter gene, a protocol was developed by which the recombinant adenoviruses could be microinjected into sciatic nerve of P10 Sprague-Dawly rats. This lead to effective transduction of Schwann cells (Figure 3). Five days after injection, nerves were excised and subjected to

immunohisochemical analysis. The distribution of epitope-tagged adenoviral-derived PMP22 was observed relative to markers for various Schwann cells subcellular organelles. This system has allowed us to determine the fate of *de novo* wild-type, *Trembler*, and *Trembler-J* PMP22 in myelinating Schwann cells *in vivo*.

Figure 1. Schematic diagram of the compact myelin of a myelinated peripheral nerve fibre. One myelinated nerve fibre (MNF) extends from a peripheral nerve (PN) of the peripheral nervous system (PNS). A series of Scwhann cells (C, cross-section) enclose the axon (A, cross-section). Each myelinating Schwann cell is surrounded by a basal lamina (BL) and forms one internode (IN) delimited by Nodes of Ranvier (NR). Myelin consists of the Schwann cell extended membrane structure that wraps the axon (CM, cross-section). The outermost myelin wrap opens to form the outer mesaxon (OM) while the innermost wrap opens to form the inner mesaxon; (IM). An enlarged illustration of the tightly associated membrane structure of compact myelin reveals a concentric biphasic pattern (CM, in black, box in foreground). The intraperiod line (IL) results from the apposition of sequential outer membranes while the apposition of the cytoplasmic faces of each wrap form the major dense line (MDL) Illustration by Joshua Colby.

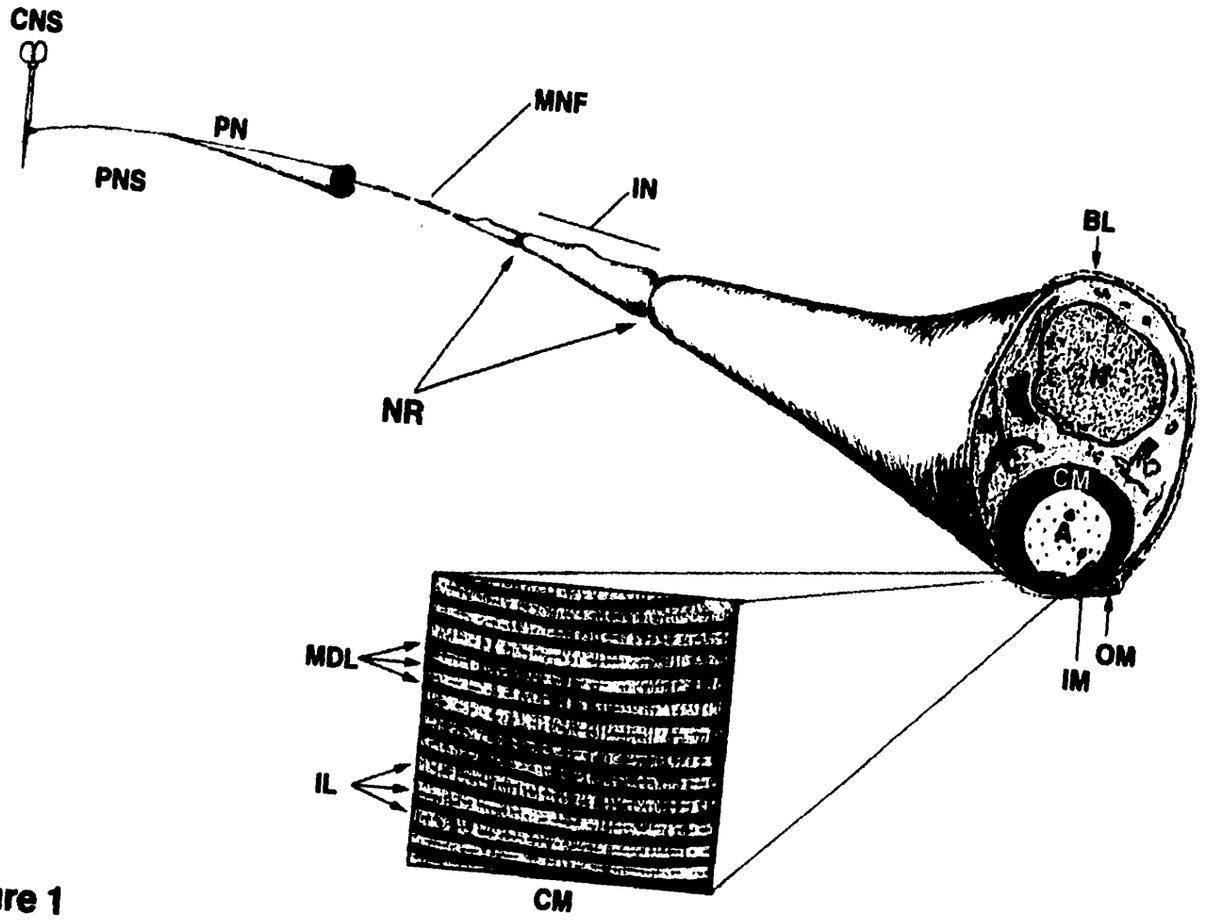

Figure 1

Figure 2. Schematic diagram of the putative structure of PMP22, a membrane tetraspan protein. PMP22 is a 160 amino-acid, 22kDa integral membrane glycoprotein. One of two extracellular domains (ext.) contains a site for N-linked glycosylation. Hydrophobicity plots predict four membrane-spanning domains (memb.). One loop between the second and third tansmembrane domain is exposed to the cytoplasm (cyto.), as well as the N-terminus and the C-terminus. The *Trembler* mutation consists of a glycine to aspartic acid substitution (trembler (G150D)) within the fourth putative transmembrane domain. The *Trembler-J* (trembler-j (L16P)) mutation consists of a leucine to proline substitution within the first putative transmembrane domain. Illustration by Kathleen Dickson.

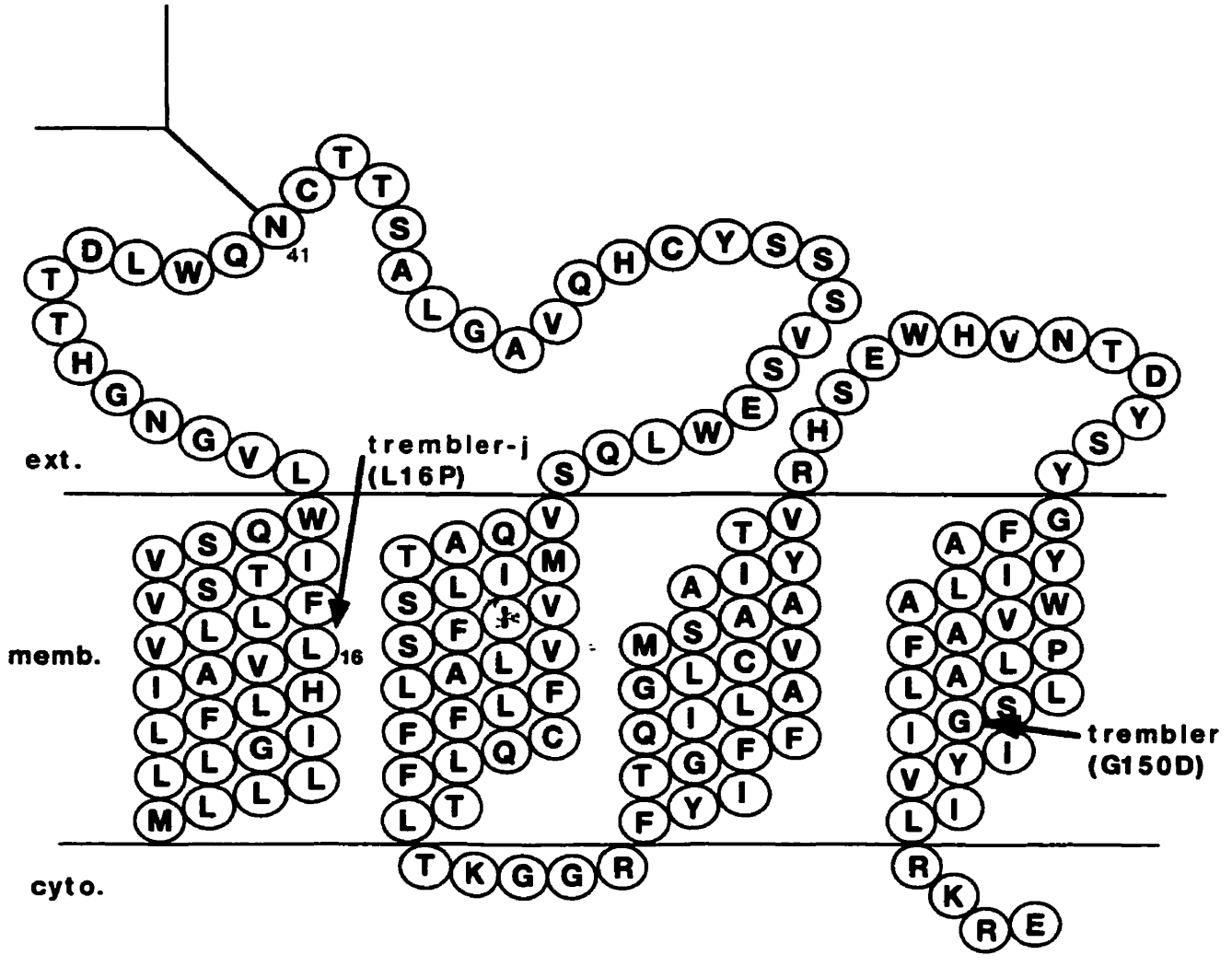

Figure 2.

Figure 3. Replication defective recombinant adenovirus expressing the *lacZ* reporter gene effectively transduces myelinating Schwann cells *in vivo*. The sciatic nerve of P10 Sprague-Dawly rats was injected with 1-2 µl *lacZ* recombinant adenovirus, excised five days later, fixed, and snap-frozen in OCT. 10 µm cryosections were stained overnight in X-gal staining buffer, stained lightly with eosin, coversliped and photographed using standard phase-contrast light microscopy. Schwann cells expressing the adenoviral derived *lacZ* reporter gene appear blue (white arrows). Some unlabeled fibres show "digestion chambers of Cajal" indicating Wallerian degeneration (black arrow). Scale bar: 20 µm.

Figure 3.

PMP22 carrying the Trembler or Trembler-J mutation is intracellularly retained in myelinating Schwann cells.

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Abstract

Peripheral Myelin Protein 22 (PMP22) is an integral membrane protein primarily localized in the compact myelin of the peripheral nervous system. In humans, a duplication or deletion of the PMP22 gene gives rise to the human hereditary neuropathies Charcot-Marie Tooth type 1A (CMT1A) or hereditary neuropathy with liability to pressure palsy (HNPP), respectively. In addition, non-conservative point mutations in the PMP22 gene cause human hereditary neuropathies as well as the naturally occurring *Trembler* (*Tr*) and *Trembler-J* (*Tr-J*) mouse models of hereditary neuropathies. Therefore, the *Tr* and *Tr-J* forms of PMP22 have provided models to study the disease mechanism of PMP22 related hereditary neuropathies. We have generated replication-deficient recombinant adenoviruses containing expression cassettes encoding VSV-G protein epitope-tagged wild-type, *Tr* or *Tr-J*PMP22 as well as the *lacZ* reporter gene. Viruses were micro-injected into the sciatic nerve of ten-day-old Sprague-Dawly rats. Injected nerves were excised five days later and subject to immunohistological analysis to determine the distribution of the virus-derived protein throughout infected myelinating Schwann cells. We report here that in a myelinating Schwann cell *in vivo*, epitope-tagged, wild-type PMP22 is successfully transported to compact myelin whereas the *Tr* and the *Tr-J*, epitope-tagged mutants are retained in the endoplasmic reticulum. This provides *in vivo* evidence that the pathogenic nature of the *Tr* and *Tr-J* mutants is most likely a function of abnormal retention within the endoplasmic reticulum of myelinating Schwann cells.

Introduction

Myelin formation in the peripheral nervous system requires the expression of structural myelin proteins, such as Protein zero (P0) (Warner *et al.* 1996) and Peripheral Myelin Protein-22 (Naef and Suter 1998), as well as regulating transcription factors such as Pax3 (Kioussi *et al.* 1995), Krox-20 (Egr2) (Warner *et al* 1998, Timmerman *et al.* 1999), SCIP (Bermingham *et al.* 1996, Jaegle *et al.* 1996), and Sox-10 (Inoue *et al.* 1999). A critical role for each of these proteins in peripheral nerve myelination has been demonstrated either by finding mutations in these proteins in humans with hereditary peripheral nerve disease or through the study of murine models of peripheral neuropathies. Despite the growing number of genes that can cause hypo- or dysmyelination in peripheral nerves, the majority of humans with hereditary peripheral neuropathies related to abnormal myelination (Charcot-Marie-Tooth (CMT) disease and related disorders) harbour mutations affecting the PMP22 gene (Naef and Suter 1998). Furthermore, among the mutations that affect the PMP22 gene, intrachromosomal duplications and deletions (as in Hereditary Neuropathy with liability to pressure palsy, HNPP) are the most common. Thus, many cases of CMT are believed to be caused by overexpression of the wild-type PMP22 protein (Roa *et al.* 1993). Similarly, the majority of cases of HNPP which result from deletion of one PMP22 allele are likely to be the consequence of underexpression of the PMP22 mRNA (Schenone *et al.* 1997) and protein (Vallat *et al.* 1996). Point mutations which give rise to mutant PMP22 protein are less common causes of hereditary peripheral neuropathies.

The first two PMP22 mutations to be identified were associated with the neuropathies in the *Trembler* (*Tr*, Suter *et al.* 1992) and *Trembler-J* (*Tr-J*, Suter *et al.* 1992) mice. Since then, the *Tr* and *Tr-J* mutant forms of PMP22 have become a paradigm for studying the molecular mechanisms underlying the PMP22-related neuropathies. The wild-type PMP22 protein is a highly hydrophobic transmembrane protein with four putative transmembrane domains (Suter and Snipes 1995). Both the *Tr* and *Tr-J* mutations introduce non-conservative amino acid changes in hydrophobic domains. The *Tr* mutation (G150D) is in the fourth hydrophobic domain, while the *Tr-J* mutation (L16P) occurs in

the first transmembrane domain. The majority of the remaining PMP22 point mutations also fall within putative transmembrane domains (Ikegami *et al.* 1998). Phenotypic differences between the heterozygous *Tr* and *Tr-J* mice and heterozygous PMP22 null mice (and humans with HNPP) clearly demonstrate that the *Tr* and *Tr-J* mutations cause peripheral nerve disease by mechanisms other than haploinsufficiency (i.e. there is a gain of function) (Adlkofer *et al.* 1997), though, as we will discuss, a contribution of haploinsufficiency to the phenotype of *Tr* and *Tr-J* mice is likely.

D'Urso *et al.* (1998), Naef *et al.* (1999) and Tobler *et al.* (1999) have reported that transiently-transfected $PMP22^{Tr}$ and $PMP22^{Tr-J}$ are retained in the ER/golgi compartment in cell lines and in primary Schwann cells. Furthermore, immunoreactive $PMP22^{Tr}$ protein was not detected in peripheral nerve myelin in compound heterozygous $PMP22^{Tr}$/PMP22-null mice though only very few myelin-like profiles were observed (Adlkofer *et al.* 1997) (Naef *et al.* 1997). In studies of heterozygous *Tr-J* mouse (*Tr-J*/+), Notterpek *et al.* (1997) reported that the presence of the mutant allele caused the marked upregulation of the endosomal/lysosomal pathway in affected nerves. In addition, treatment of the affected nerves with chloroquine resulted in the increased accumulation of several compact myelin proteins, including PMP22, MBP, and P0 when compared to normal nerves. This result led to the hypothesis that the $PMP22^{Tr-J}$ allele might be incorporated into myelin and that the presence of the mutant protein in myelin results in increased myelin turnover (Notterpek *et al.* 1997).

Myelin formation requires the interaction of axons and Schwann cells. Although PMP22 expression can be modulated *in vitro* in the absence of axons, robust myelin PMP22 mRNA and protein expression is only observed *in vivo* (Suter *et al.* 1994). Many studies have documented the marked downregulation in myelin gene expression when axons are lost (i.e. during Wallerian degeneration) and the marked upregulation of myelin gene expression during axon regeneration (see Snipes *et al* 1992 and references therein). Myelination may also be regulated by post-transcriptional mechanisms that are also axon dependent. For example, recent experiments have suggested that axonal contact facilitates

the translocation of PMP22 from the golgi apparatus to the plasma membrane of Schwann cells where it is incorporated into myelin (Pareek *et al.* 1997).

Thus, while *in vitro* studies strongly suggest that the *Tr* and *Tr-J* mutations are retained in the endoplasmic reticulum/golgi compartments, *in vivo* studies suggested the alternative hypothesis, namely, that mutated PMP22 isoforms might be incorporated into myelin where they exert their deleterious effects. This hypothesis is all the more plausible since, as cited above, there is evidence that the intracellular trafficking of myelin proteins in Schwann cells may be dependent on axonal contact. To resolve the question of the fate of mutant PMP22 protein *in vivo*, we have constructed a series of replication-defective adenoviruses which can deliver either epitope-tagged wild-type or mutant PMP22 variants into myelinated nerves so that the intracellular fate of these PMP22 proteins could be determined. Furthermore, to track the infections with these viral constructs, the wild-type and mutant PMP22 cDNAs were made bicistronic with the *LacZ* reporter gene. We found that the epitope-tagged wild-type PMP22 is transported throughout the Schwann cell cytoplasm and colocalizes with myelin. The epitope-tagged $PMP22^{Tr}$ and $PMP22^{Tr\text{-}J}$ proteins, on the other hand, are retained in the ER network of myelinating Schwann cells and are not associated with myelin.

Materials and Methods

DNA subcloning

We generated a general purpose cloning vector, pBS CMV polio-BP/lacZ, for expressing PMP22 cDNAs bicistronically with β-galactosidase. This construct incorporates (from 5' to 3): the CMV promoter/enhancer from pCEP4 (Invitrogen), most of the polylinker from pBluescript KS+, the internal ribosome entry site from poliovirus (Nicholson *et al.* 1991), a synthetic oligonucleotide (5' AGCTTG ACATCA TAGAAG CACTCT ACTATA TTCATT CCGGCA AAAACC GGTAC-3') encoding a strong bacterial promoter/enhancer (BP), and a *LacZ* with the SV40 polyadenylation signal from pSV-β-Galactosidase (Control Vector, Promega). Additionally, this construct was flanked

by *SalI* restriction sites to facilitate subcloning into the transfer vector, pShuttle described by He (He *et al.* 1998). Details of the subcloning are available on request (jsnipes@mni.lan.mcgill.ca).

PMP22 constructs were subcloned as follows: The *HindIII/ApaI* fragment from pcDNA1TrVSV (Naef *et al.* 1997) containing $PMP22^{Tr}$ with a 3' VSV-G epitope tag and a *HindIII/EcoR1* fragment from pcDNA1Tr-JVSV containing $PMP22^{Tr\text{-}J}$ with a 3' VSV-G epitope tag (Naef *et al.* 1997) were subcloned into the *HindIII/* site of pBlueScript II KS+ (Stratagene). Wild-type PMP22 VSV was recreated by replacing a *Pst1* fragment containing the *Tr* mutation in pBS TrVSV with the corresponding, non-mutated, *Pst1* fragment from pBS Tr-JVSV to create pBS wtVSV. *XhoI/NotI* inserts were removed from pBS wtVSV, pBS TrVSV and pBS Tr-JVSV and directionally ligated into the polylinker of pBS CMV polio-BP/lacZ downstream of the CMV promoter and upstream of the IRES and *lacZ*. The entire expression cassette was cut with *SalI*, gel purified, and ligated into the *SalI* site in the shuttle vector (pShuttle) (He *et al.* 1998). Each shuttle vector containing the PMP22 expression cassettes was linearized with *PmeI* and gel purified (Qiaex, Qiagen, Inc). 30 ng of linearized, purified PMP22 shuttle vector was mixed with 300 ng of the cosmid, pAdEasy-1, and electroporated into 25 µl electrocompetent BJ5183 *E. Coli*, as described (He *et al.* 1998). To ensure DNA stability, cosmid DNA from selected recombinant clones was electroporated into electroMAX DH10B cells (Life Technologies). The presence of intact wild-type and mutated PMP22 cDNA in the adenovirus vectors was confirmed by automated sequencing. A control recombinant adenovirus was constructed that contains the CMV promoter, IRES, bacterial promoter/enhancer, and *LacZ*, but no PMP22 coding sequence.

Cell Culture and Adenoviral Infections

Primary human embryonic kidney cells (QBI-293A) containing the E1A and E1b region of human adenovirus type 5 (Quantum Biotechnologies, Inc.) were maintained in DMEM (Bio-Whitaker), supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine (Gibco BRL), 1% Antibiotic/Antimycotic (contains penicillin, streptomycin

sulfate and amphotericin B, Gibco BRL) at 37°C in a humidified incubator containing 5% CO_2. 293 cells were infected with adenovirus in the supplemented DMEM for 90 minutes at 37°C, unless otherwise noted.

Production of Adenoviruses

Five micrograms of *PacI* linearized, ethanol precipitated, adenoviral DNA was transfected into $1.5x10^6$ 293A cells using a standard $CaPO_4$ technique (Ausebel *et al.* 1989). After 5-7 days, the cells and supernatant were subjected to three freeze-thaw cycles (methanol/dry-ice bath to 37°C water-bath), followed by centrifugation at 2000 rpm for 10 minutes in a clinical centrifuge (IEC Centra CL). The supernatant was removed, added to an equal volume of fresh media and placed on 1 x 10^6 293A cells in a 25 cm^2 flask 37°C, for 3 hours. Fresh media was added, the cells were incubated for 72 hours, harvested, lysed as above, and the supernatant used to re-infect 5 x 10^6 293A cells in a 100 mm tissue culture dish. The final round of amplification consisted of $2.0x10^8$ 293A cells 100% transformed and harvested 72 hours post-infection. After five freeze/thaw cycles, cell debris was removed by centrifugation. The virus was further purified by discontinuous (1.2M/1.4 M) cesium chloride gradient centrifugation (22,500 rpm, Beckman 50.2 Ti rotor, 2 hrs) followed by a second cesium gradient (1.2M/1.4) centrifugation (30,000 rpm, Beckman SW60 rotor, 55 min) and dialyzed overnight at 4°C against 10 mM Tris, 10 mM $MgCl_2$, 10% glycerol, pH 7.4. All viruses were stored at -80^0C prior to use.

Quantitation of Adenoviruses

To quantify the gene transfer unit (GTU) concentration of purified viruses, serial dilutions of viral stock were used to infect 1 x 10^6 293A cells plated in 35 mm gridded tissue culture dishes. Cells were incubated 14 hours, rinsed with PBS (20 mM NaH_2PO_4, 150 mM NaCl), fixed 30 minutes in 2% paraformaldehyde/PBS, rinsed 10 minutes in 0.1M phosphate buffer, pH 7.4, 0.01% sodium deoxycholate, 0.02% Igepal CA630-(Sigma), and then stained overnight in 0.4 mg/ml X-gal (GibcoBRL, as described by Foran and Peterson (1992)). Blue cells per grate were counted to calculate GTU/ml of virus stock.

Immunoprecipitation of PMP22

One million 293A cells were infected with adenovirus at an multiplicity of infection (m.o.i) of 10. After infection, the cells were incubated at 37°C for 13 hrs, rinsed twice with PBS, and incubated in ^{35}S methionine (0.1mCi/ ml Trans-^{35}S label, ICN Pharmaceuticals) in methionine-free DMEM supplemented with L-glutamine. After a 3 hr incubation, the labeling medium was removed and the cells were rinsed twice with ice-cold PBS, and once with ice-cold 200 mM NaCl, 50 mM HEPES, pH 7.6. Immunoprecipitations were carried out essentially as previously described (Pareek *et al.* 1993) using 10 µl of rabbit anti-mouse PMP22 (against the murine homologue of rat peptide 2, (Snipes *et al.* 1992)) per sample except that the immunoprecipitates were treated at 65°C immediately prior to electrophoresis. Proteins were resolved on 15% polyacrylamide gels and processed for fluorography (Enhance, NEN Dupont) according to the manufacturer's instructions.

Injection of recombinant adenoviruses into rat sciatic nerves.

All injections were carried out using a Drummond Nanoject automatic injector (Drummond Scientific) mounted on a Taurus manual micromanipulator (WPI). Microinjection pipettes were prepared from 3.5 inch glass tubes (part # 3-00-203-G/X, Drummond) using a P-77 Brown-Flaming Micropipette Puller (Sutter Instrument Co. San Francisco) to a minimum outer diameter of approximately 40 µm. Micropipettes were filled with sterile mineral oil and then loaded with 3-4 µl of aqueous viral stock. Ten day old Sprague-Dawley rats (Charles River, Canada) were anaesthetized using 7.5 µl/g body weight avertin (40.4 mg/ml 2,2,2 tribromoethanol and 2.5% tert-amyl alcohol in water, Aldrich) and placed on a chilled pad. An incision was made to expose the right sciatic nerve at the mid-thigh level. Virus was injected over a 5 mm segment of the sciatic nerve in 46 nl volumes to minimize sudden pressure changes, for a total of 1-2 µl virus into 3-4 injection sites. Incisions were closed using 3.0 silk sutures (Ethicon) and Vetbond (3M Animal care products, MN), then warmed under a heat lamp. Nine animals were injected unilaterally per viral construct. Five days after injection, animals were euthanized with 15

mg ketamine (i.p.; Ayers), the sciatic nerves were removed and fixed in 4% paraformaldehyde/PBS at 4°C for 3 hours. Nerves were cryoprotected overnight at 4°C in 30% sucrose, embedded in OCT (Tissue-Tek) and frozen in isobutanol/dry ice (-30°C). Ten micron thick frozen sections of the rat sciatic nerves were prepared using a Micron HM 500M cryostat and stored at –80°C. At least one serial section from all injected nerves was stained with X-gal and counterstained with eosin to assess efficacy of infection.

Immunofluorescence: tissue sections

All antigens were visualized by indirect immunofluorescence. For tissue sections, all procedures following the cryosectioning were performed at room temperature. Tissue sections post-fixed in 4% paraformaldehyde/PBS (20 mM NaH_2PO_4, 150 mM NaCl) for 10 minutes, and immunostained as described by (Naef *et al.* 1997) with the following modification, primary and secondary antibodies were diluted in a blocking buffer consisting of PBS/0.2% Tween-20/ 10% milk. Primary antibodies included rabbit anti-VSV G-protein antiserum ((Naef *et al.* 1997); 1:1000), mouse monoclonal anti-MBP antibody (Boehringer Mannheim; 1:250), and mouse monoclonal anti-protein disulphide-isomerase (anti-PDI, clone RL-77 Affinity Bioreagents, Inc; 1:200); Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch; 1:300) and FITC-conjugated goat anti-mouse antibody (Jackson Immunoresearch; 1:200) were used as secondary antibodies. Non-immune rabbit serum was used as a control for the anti-VSV antibody. In some sections, nuclei were stained with Hoechst dye 33258 (Pierce) diluted 1:50,000 in PBS/0.2% Tween-20 for 15 minutes. Sections were mounted with Immuno Floure Mounting Media (ICN). Immunostained sections were visualized by standard fluorescence microscopy (Zeiss Axioskop) or by laser-scanning confocal microscopy (Zeiss 410 LSM) using standard filter sets.

Immunofluorescence: cell culture

Seventy thousand 293A cells were plated on 12 mm coverslips (Carolina, Germany) and grown overnight in DMEM supplemented as above. The cells were infected at 37°C for 1.5 hr at an m.o.i =10 and then rinsed in PBS. Twelve hours later, the cells were fixed in 95% ethanol/5% glacial acetic acid for 15 min at –20°C. The cells were washed 3 times in PBS and incubated in blocking solution (2.5 mM Tris, pH , 137 mM NaCl, 2.7 mM KCl (TBS)) /1% triton X-100, 2% BSA, 0.1% porcine skin gelatin, 10% normal goat serum and 2% non fat dried milk). The anti-VS-G serum was absorbed against a liver acetone powder and incubated with the cells for 2 hrs at room temperature (final dilution 1:1000, anti-PDI (1:200) in blocking solution). The cells were washed 3 x 15 min in TBS/0.2% Tween-20 and then incubated with the appropriate secondary antibodies and visualized by standard fluorescence or laser confocal microscopy as described above.

Results.

Design, preparation, and characterization of recombinant adenoviruses expressing PMP22.

We synthesized a series of recombinant replication-defective adenoviruses as shown schematically in Figure 1A to examine the intracellular processing of wild-type and mutant PMP22 *in vivo*. We designed two features into our constructs to facilitate the interpretation of our results. First, we had to be able to specifically track the wild-type and mutant PMP22 variants in a background which contained the abundant endogenous PMP22 protein that is present in peripheral nerve myelin. To do this, we utilized a VSV epitope tag at the carboxy-terminus of the wild-type PMP22, PMP22^{Tr}, and PMP22$^{Tr\text{-}J}$ proteins. Naef *et al* (Naef *et al.* 1997) had previously characterized the VSV tagged PMP22 and showed that the epitope-tagged wild-type protein could be incorporated into the plasma membrane of Schwann cells *in vitro*. Secondly, we made a bicistronic expression cassette for epitope-tagged PMP22 and β-galactosidase by placing the ribosome entry site isolated from the polio virus downstream of the epitope-tagged PMP22 cDNAs,

but upstream of the LacZ gene and a poly A tail. Thus, β-galactosidase activity could be used as a semi-quantitative monitor for the expression of the PMP22 protein since both are translated from the same mRNA.

We performed immunoprecipitations to confirm that the viruses efficiently expressed the wild-type and mutant PMP22 proteins in 293 cells. Figure 1B shows that anti-mouse PMP22 peptide-2 antibodies precipitate PMP22 from 293 cells infected with the PMP22 constructs, but not from 293 cells infected with an identical adenovirus that expresses β-galactosidase but lacks the PMP22 cDNA. Histochemical staining of parallel cultures of 293 cells for β-galactosidase activity revealed efficient adenoviral infection of the 293 cells (not shown). Finally, we used indirect immunofluorescence employing the anti-VSV epitope antiserum to verify the subcellular localization of PMP22 constructs when infected into 293 cells. As expected, *in vitro*, epitope-tagged wild-type PMP22 could be detected on the plasma membrane, but neither the epitope-tagged $PMP22^{Tr}$ nor $PMP22^{Tr\text{-}J}$ was found on the cell surface, being localized predominantly in the ER compartment (Figure 2) as revealed by colocalization experiments for the epitope-tagged PMP22.

Adenovirus efficiently delivers PMP22 to Schwann cells in vivo without causing significant demyelination.

Overexpression of PMP22 or expression of mutant PMP22 proteins have been associated with demyelination /dysmyelination. Thus, to evaluate the trafficking of PMP22 to myelin, it was important to first determine whether adenoviral delivery of PMP22 under the control of the CMV promoter would produce such a degree of myelin loss so that it would be difficult or impossible to evaluate whether the PMP22 mutations were incorporated into the myelin sheaths of Schwann cells *in vivo*. To check this, we infected normal rat sciatic nerves with the mutant and wild-type PMP22 adenoviral constructs. We then stained the nerves for β-galactosidase activity to identify the infected cells. After 5 days of infection, we observed that the majority of *LacZ* positive cells were associated with apparently intact myelin sheaths (data not shown). We did not detect any fibers that

showed acute myelin loss in cryosections that resembled the *Tr* or *Tr-J* nerves which show many thinly myelinated axons, though this was not rigorously evaluated in 1 μm epon sections. However, a significant number of unlabeled fibers showed "digestion chambers of Cajal" indicating active axonal (Wallerian) degeneration. At least part, and perhaps most, of the axonal injury is secondary to mechanical injury to axons at the site of the microinjection of adenovirus since significant Wallerian degeneration was also present in vehicle-injected control nerves. Despite the Wallerian degeneration, the remaining myelinated fibers were sufficiently intact to allow us to trace the intracellular distribution of tagged PMP22 constructs in sciatic nerves, *in situ*.

Epitope-tagged wild-type PMP22 is incorporated into myelin *in vivo*.

Previous studies had determined that epitope-tagged wild-type PMP22 could be transported to the plasma membrane of Schwann cells *in vitro* (Naef *et al.* 1998; D'Urso *et al.* 1998), but it was unclear whether the epitope tagged protein could be incorporated into myelin, *in vivo*. To test this, we used indirect immunofluorescence to detect the distribution of the VSV epitope-tagged wild-type PMP22 with anti-VSV antibodies. Figure 3 shows that VSV-tagged wild-type PMP22 is distributed along the length of the myelinating Schwann cell parallel to the axon. This distribution could correspond either to VSV tagged PMP22 having been incorporated into myelin or alternatively, the VSV tagged PMP22 could be distributed along the adaxonal Schwann cell cytoplasm adjacent to the myelin sheath. To distinguish between these two possibilities, we performed double-label immunofluorescence for the VSV tagged PMP22 and for myelin basic protein (MBP), a major protein component of compact myelin. In addition, we used laser-scanning confocal microscopy to produce optical sections of approximately 1 μm thickness to minimize artifactual colocalization. In a subset of fibers, as shown in panels a-f of figure 3, we identified several profiles in which the VSV-tagged PMP22 immunofluorescent signal overlapped with the extreme outer portions of the myelin sheath. Whether this reflected preferential incorporation of newly synthesized PMP22 into the outer portions of previously formed myelin, or localization of the recently synthesized PMP22 to the abaxonal Schwann cell cytoplasm could not be easily resolved. In another subset of fibers,

as shown in panels g-l of figure 3, we could unambiguously identify a number of profiles in which the distribution of the VSV-tagged wild-type PMP22 colocalized with myelin basic protein by immunofluorescence. To summarize, newly synthesized epitope tagged wild-type PMP22 protein is efficiently transported along the length of myelinating Schwann cells where at least some of it appears to be incorporated into compact myelin.

Epitope-tagged PMP22^{Tr} and PMP22$^{Tr\text{-}J}$ are not incorporated into myelin *in vivo*.

Having verified that epitope-tagged wild-type PMP22 is efficiently transported throughout the Schwann cell and colocalizes with myelin basic protein, we then tested whether the *Tr* and *Tr-J* mutant forms of PMP22 were also associated with myelin. Accordingly, we injected the sciatic nerves of rat pups with adenovirus carrying wild-type and mutant forms of PMP22. After 5 days the nerves were excised and examined by confocal microscopy for the distribution of the epitope-tagged PMP22 proteins. As shown in Fig 4 , both the *Tr* and *Tr-J* mutations were retained in an antiparallel intracellular compartment that was centered around a negatively-stained oval structure (the Schwann cell nucleus, see below) and extended bidirectionally parallel to the axon with a slight spiral for a small portion of the internode. Double-labeling for the VSV epitope and myelin basic protein demonstrated that the VSV-tagged mutant PMP22-containing structure spiralled around the myelin basic protein immunoreactive myelin sheath confirming that this intracellular compartment was associated with Schwann cells. In contradistinction to the wild-type PMP22 protein, colocalization of the epitope-tagged mutant PMP22 and MBP was not observed. Thus the epitope-tagged PMP22 carrying the *Tr* and *Tr-J* mutations are not incorporated into the myelin sheath.

PMP22^{Tr} and PMP22$^{Tr\text{-}J}$ accumulate in the ER compartment of myelinating Schwann cells.

Based on the morphology of the Schwann cell compartment that contained the mutant PMP22 proteins and prior work showing that these protein are retained in the endoplasmic reticulum *in vitro*, we performed triple-label immunofluorescence for the

VSV epitope (Cy3-red), protein disulphide isomerase (fluorescein-green) and Hoechst dye 33258 (blue) to identify the intracellular compartment containing the epitope-tagged mutant PMP22 proteins. As shown in figure 5, the epitope-tagged mutant PMP22 colocalizes with the endoplasmic reticulum resident protein, protein disulphide isomerase. Furthermore, the ER compartment was centered around the oval Schwann cell nuclei visualized by the Hoechst dye, forming the same loose spiralling pattern that we observed around the myelin sheath immunostained for MBP (figure 5).

Discussion.

We developed a series of adenoviral vectors to test whether PMP22 carrying the *Tr* (G150D) and *Tr-J* (L16P) mutations is incorporated into the myelin sheath produced by Schwann cells in the peripheral nervous system *in vivo*. Upon delivering these vectors to peripheral nerves, we found that the epitope-tagged wild-type PMP22 becomes colocalized with myelin basic protein whereas epitope-tagged PMP22 carrying either the *Tr* or *Tr-J* mutations are retained in a predominantly ER-type localization. These findings have implications in the context of the biology of PMP22, our understanding of hereditary peripheral nerve diseases, the cellular mechanisms of PMP22 mutations, and the use of adenovirus as a vehicle to deliver genes to intact myelinating Schwann cells *in vivo*.

There is a growing number of missense mutations that have been identified in the PMP22 gene that lead to hypomyelination or demyelination of peripheral nerves in *Trembler* and *Trembler-J* mice and in humans with Charcot-Marie-Tooth disease, and Dejerine-Sottas syndrome (Naef and Suter 1998). These mutated proteins could affect myelin formation through one of several major mechanisms. The abnormal protein could interfere with myelin via abnormal protein-protein or protein-lipid interactions within the nascent or mature myelin sheath. Alternatively, since the *Tr* and *Tr-J* mutations are non-conservative, the abnormal protein could be retained in the ER and degraded by ER quality control mechanisms. Although transient transfection studies in isolated Schwann cells (D'Urso *et al.* 1998) (Naef and Suter 1999) and the adenoviral infection into intact myelinated nerves strongly support the ER retention mechanism for many of the mutations,

it is expected that the resulting phenotype would result from haploinsufficiency unless the mutations interfered with additional cellular processes. Haploinsufficiency of PMP22 is well-modelled by humans with hereditary neuropathy with liability to pressure palsies(Chance *et al.* 1993) and by mice with heterozygous null PMP22 alleles (Adlkofer *et al.* 1997). Humans and mice carrying the L16P (*Tr-J*) and G150D (*Tr*) mutations appear to result from "gain of function" mutations since they have clinical and pathologic phenotypes that clearly differ from those generated by haploinsufficiency (Snipes *et al.* in press).

As outlined in the introduction, there were many reasons for cautious interpretation of studies concerning the biosynthesis and targeting of myelin proteins in the absence of myelin formation. First, myelin formation is a complex process that involves critical interactions between axons and the ensheathing Schwann cells (Scherer 1997). Our original observations suggested that wild-type PMP22 was not efficiently targeted to the plasma membrane in the absence of axons and myelin formation indicated that axonal signals regulate the intracellular distribution of PMP22, at least from the golgi to the plasma membrane (Notterpek *et al.* 1997). Secondly, our finding that most of the newly synthesized wild-type PMP22 is not processed beyond the ER and is degraded with a half-life of 45 min to 1 hr also were consistent with the hypothesis that successful PMP22 biosynthesis through the golgi and into the plasma membrane might critically depend on the presence of axon-derived signals (Pareek *et al.* 1993). Finally, our studies of the *Tr-J* neuropathy led to the hypothesis that mutant PMP22 carrying the *Tr-J* mutation (L16P) was incorporated into myelin leading to increased myelin turnover and increased degradation of PMP22 as well as other myelin proteins in the lysosomes (Notterpek *et al.* 1997). Nevertheless, our *in vivo* results confirm previous *in vitro* studies (Naef *et al.* 1997; D'Urso *et al.* 1998; Naef and Suter 1999) demonstrating that the *Tr* and *Tr-J* mutations are not incorporated into the plasma membrane of Schwann cells and are not incorporated into myelin.

Several of the above-mentioned hypotheses can be re-evaluated in light of findings reported here and other recent studies. First, based on immunofluorescence results, it

appears that wild-type PMP22 can be transported to the plasma membrane of non-myelinating Schwann cells and COS cells in the absence of axonal contact. Even during myelination, this likely represents only a small percentage of the newly synthesized PMP22 as only a small percentage of PMP22 becomes complex glycosylated in the golgi apparatus (Pareek *et al.* 1997). Previous studies failed to recognize small quantities of PMP22 on the cell surface of Schwann cells probably because of limitations in the techniques and/or the sensitivity of the antibodies used for detection (Pareek *et al.* 1993). Secondly, our previous hypothesis that $PMP22^{Tr\text{-}J}$ is incorporated into myelin appears to be incorrect. This conclusion was based on the finding of increased lysosomal degradation of PMP22, MBP and P0 in *Tr-J* animals (Notterpek *et al.* 1997). Based on our present findings, the most likely interpretation is that the increased lysosomal degradation of these proteins reflects increased myelin turnover secondary to either haploinsufficiency of PMP22 and/or a likely "gain of function" effect in the endoplasmic reticulum (Naef *et al.* 1997, Naef and Suter 1998, Naef *et al.* 1997) of myelinating Schwann cells.

The PMP22 *Tr* and *Tr-J* mutations are members of a growing class of disease-causing mutations including those causing alpha-1-antitrypsin deficiency (Poller *et al.* 1999), cystic fibrosis (Jiang *et al.* 1998), murine obesity (Jackson *et al.* 1997), congenital goiter and hypothyroidism (Kim *et al.* 1996), sucrase-isomaltase deficiency (Ouwendijk *et al.* 1996), and glycogen storage disease, type 1a (Lei *et al.* 1994) among others that are recognized by retention of the mutant proteins in the endoplasmic reticulum. All cells have intrinsic quality control mechanisms operative in the ER to ensure proper protein folding, sorting, and perhaps oligomerization. As a transmembrane glycoprotein, likely containing intracellular disulphide bonds, PMP22 is predicted to interact in the ER with proteins such as calnexin (Bergeron *et al.* 1998), calreticulin (Zhang *et al.* 1997), Erp57 (Elliott *et al.* 1997) and/or other chaperone proteins. Abnormal or saturating interactions with any of these proteins could explain the "gain of function" phenotype of the PMP22 *Tr* and *Tr-J* mutations. Naef *et al* have recently demonstrated that many of the PMP22 point mutations are likely to be "ER retention" mutations (Naef and Suter 1999). Accordingly, saturating interactions with ER resident proteins, although less likely because of the large volume of the ER and the large relative abundance of ER proteins, could be a disease mechanism for

the majority of cases of Charcot-Marie-Tooth disease, type 1A that are caused by overexpression of the wild-type PMP22 protein.

Mutations in several genes including PMP22, MPZ (protein zero), GJβ-1 (connexin 32), EGR-2, (early growth response-2, also known as Krox-20), and SOX10 (Inoue *et al.* 1999) have now been associated with hereditary sensory and motor neuropathies. One can ask whether ER retention is a common mechanism among this class of diseases. Extrapolating from *in vitro* data (D'Urso *et al.* 1998, Naef and Suter 1999, Tobler *et al.* 1999), which we have validated for the *Tr* and *Tr-J* mutations *in vivo*, it appears likely that many PMP22 mutations result in abnormal ER retention/degradation of the PMP22 protein. ER retention is also likely to be a mechanism in a subset of the MPZ mutations (Orfali and Snipes in press), though loss of function or other types of gain of function (i.e. not related to ER retention) are more probable with the vast majority of MPZ (adhesion), GJβ-1 (gap junctions), EGR2 (transcriptional regulation), and SOX10 (transcriptional regulation) mutations.

Because of the importance of axon-glial interactions (Bunge 1987), it has proven difficult to study processes which may be unique to myelination. One approach is to study myelination in neuron-glial cocultures under myelinating conditions (Eldridge *et al.* 1987). Myelinating co-cultures are technically difficult to set-up, myelination occurs over a protracted time, and the efficiency of myelination *in vitro* as measured by thickness of myelin appears reduced (Notterpek *et al.* 1999) though this can be partially ameliorated by addition of progesterone to the culture medium (Koenig *et al.* 1995) (Notterpek *et al.* 1999). A second approach to the study of myelination is through transgenic mice and knock-out technologies (Snipes and Suter 1995). As powerful as these techniques are, they are expensive and, until recently with the advent of inducible and targeted expression of transgenes (e.g.Chen *et al.* 1998), it has been difficult to achieve controlled expression of transgenes. Replication-defective adenovirus has a number of positive attributes that make it a suitable candidate as a gene delivery vector to study myelination in the peripheral nervous system (Slack and Miller 1996). Thus, we have chosen to use adenovirus as a vehicle for delivering genes *in vivo* into intact and forming sciatic nerves. Our results,

together with those of Shy (Shy *et al.* 1995) and Guenard (Guenard *et al.* 1999) demonstrate that adenovirus can effectively transduce Schwann cells *in vivo*. In addition, using our experimental approach, it is now possible to determine the effects of single mutations in myelin genes on intracellular targeting and on myelin formation *in vivo*. Indeed, (Guenard *et al.* 1999) *et al.* have reported that adenovirus can sustain stable expression of β-galactosidase for up to two months in Schwann cells *in vivo*. We have also observed strong expression of our β-galactosidase reporter which is bicistronic with our PMP22 expression cassettes (data not shown). Although there are indications of post-transcriptional regulation of PMP22 expression (Bosse *et al.* 1999), the detection of the β-galactosidase can be taken as evidence for PMP22 mRNA expression except of our control virus lacking PMP22 cDNA.

Unlike retroviruses, adenoviruses can infect nonproliferating cells which is important when studying differentiated cells such as myelinating Schwann cells. For the experimental conditions reported here, there is little evidence that adenoviral expression interferes with the function of Schwann cells as myelin forming cells. However, we noted significant Wallerian degeneration which is, at least partially, due to mechanical injury at the site of injection. We cannot, however, rule out the possibility that the adenovirus itself is causing acute axonal damage. We (data not shown), and others (Guenard *et al.* 1999), have also observed a pronounced chronic inflammatory neuritis after long-term exposure of nerves to the adenovirus. Immunosuppressive therapy, such as with FK506 has been shown to reduce adenoviral-induced inflammation in other tissues (Lochmuller *et al.* 1995) and has been used to increase the duration of adenovirally-delivered transgenes in Schwann cells *in vivo* (Guenard *et al.* 1999).

In summary, we have validated an adenovirus model for studying the effects of mutations in myelin proteins *in vivo*. We have used this model to demonstrate that PMP22 carrying the *Tr* and *Tr-J* mutations are retained in the ER and is not incorporated into the myelin sheath. Thus, the adenoviral system appears to be a powerful system for analyzing the effects of myelin gene mutations *in vivo*.

Acknowledgements

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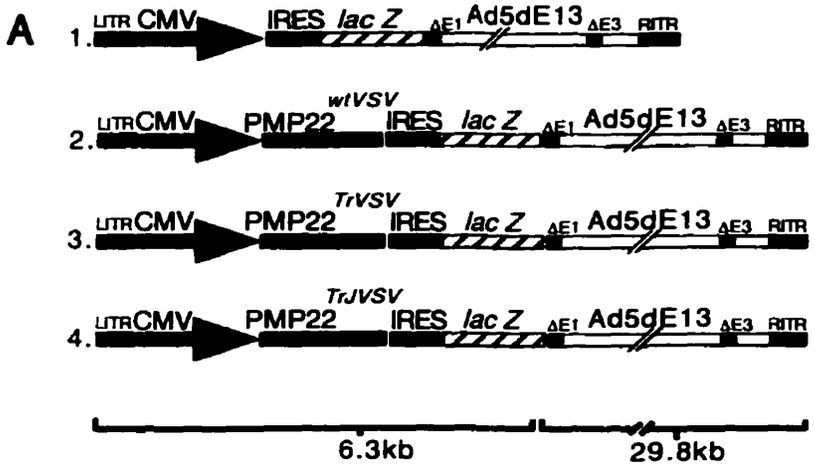
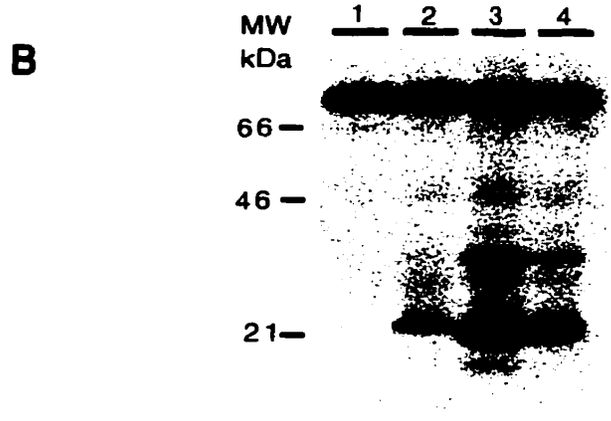
Manuscript Figures

Figure 1a. Schematic diagram of the four recombinant adenoviral constructs used in this study. Recombined into the 33 Kb Ad5ΔE1ΔE3 genome, a bicistronic expression cassette is driven by the CMV promoter and contains PMP22*wt* (2), PMP22*Tr* (3), or PMP22*Tr-J*(4), each with a 3' VSV G-protein epitope tag, followed by an IRES, the *lacZ* reporter gene and a poly A tail (not shown). The same expression cassette lacking a PMP22 insert was used to generate a l*acZ* expressing recombinant adenovirus (1).

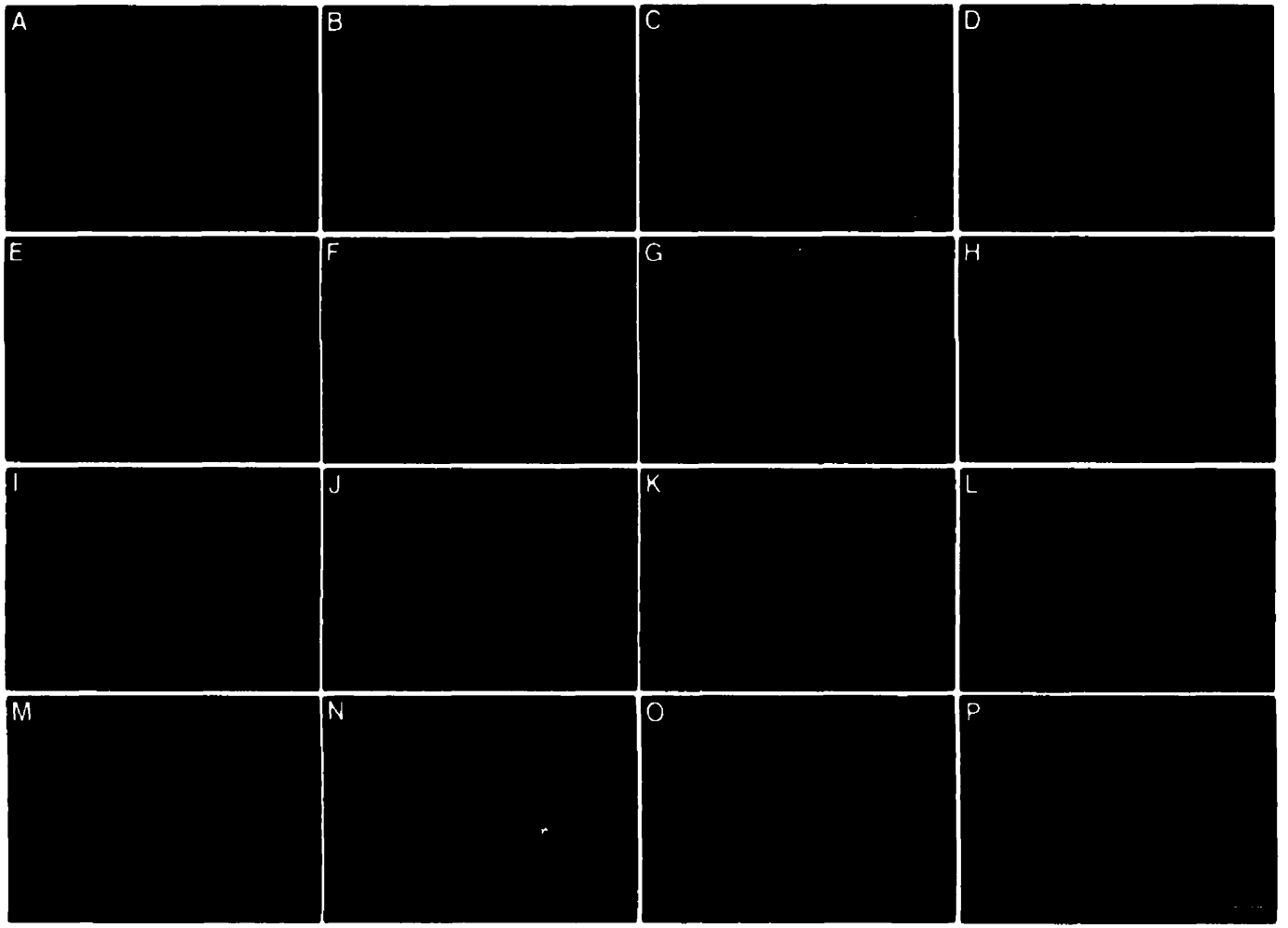
Figure 1b. Recombinant adenoviruses produce PMP22 protein in mammalian cells. One million 293A cells were infected with the *lacZ, wtVSV, TrVSV,* or *TrJVSV* recombinant adenoviruses (MOI=10), incubated 13 hours, then incubated for 3 hours in medium containing 0.1mCi/ml Trans-^{35}S to label newly synthesized protein. Cells were harvested and 50 μg whole protein was subject to immunoprecipitation using a rabbit anti-mouse PMP22 antibody. Resolved on a 15% polyacrylamide gel and subject to fluorography, immunoprecipitates from cells infected with *wtVSV*(lane 2), *TrVSV*(lane 3), and *Tr-JVSV*(lane 4) adenovirus produced PMP22 whereas control *lacZ*-infected cells did not (lane 1).





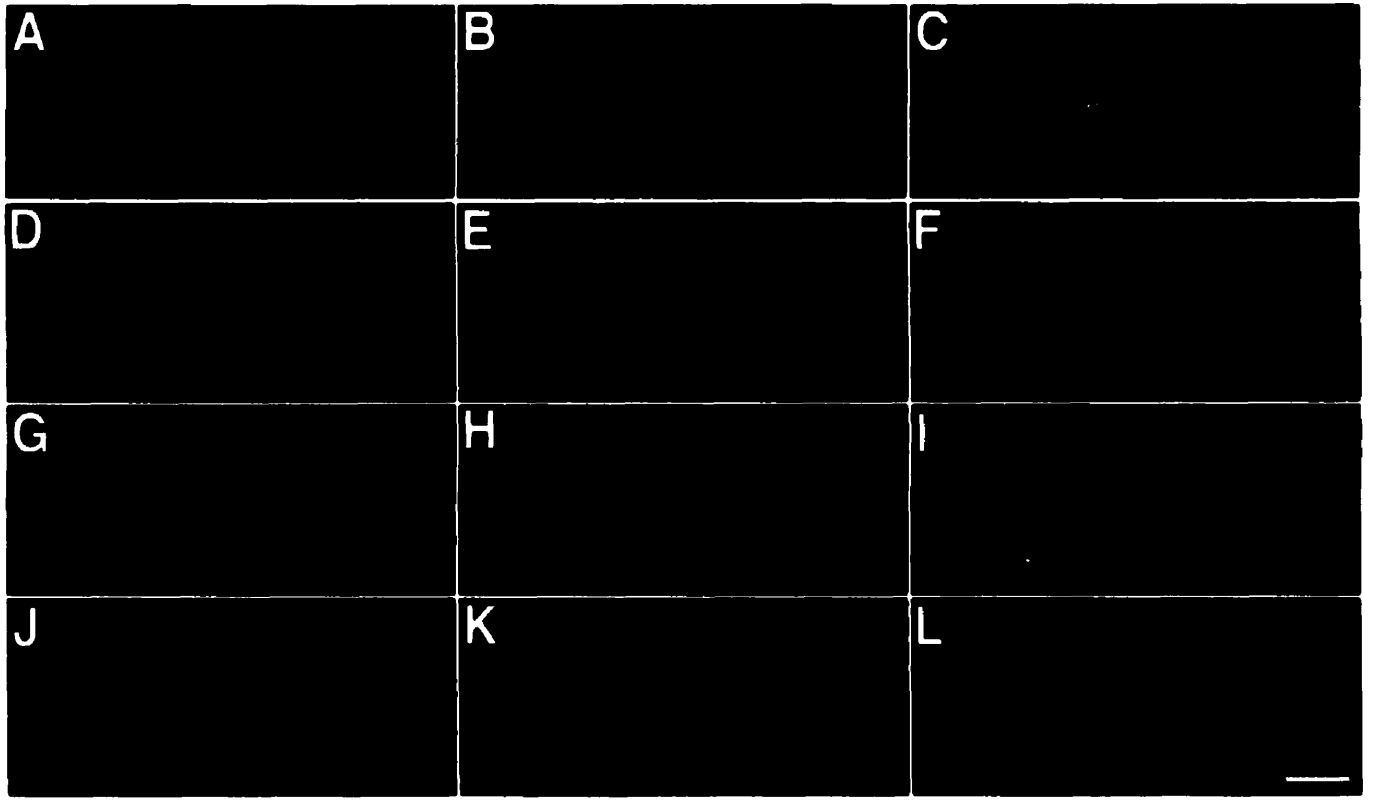
Manuscript Figure 1.

Figure 2. PMP22^{wtVSV} is transported to the membrane of 293A cells whereas PMP22^{TrVSV} and PMP22$^{Tr\text{-}JVSV}$ remain primarily in the ER. 293A cells grown on coverslips were infected with PMP22*wtVSV* (panels a-e), PMP22*TrVSV* (panels e-h), PMP22*Tr-JVSV* (panels i-l), or *lacZ* (panels m-p) recombinant adenovirus, incubated 12 hours, fixed and subject to immunohistochemical analysis. Cells were double labelled using a rabbit polyclonal anti-VSV G-protein antiserum conjugated to a Cy3 conjugated anti-rabbit secondary antibody (panels a,e,i,m), and a mouse monoclonal anti-PDI primary antibody conjugated to FITC-conjugated anti-mouse immunoglobulin secondary antibody (panels b,f,j,n)(overlays shown in panels c,g,k,o). Non-immune rabbit serum was used as a control for the anti-VSV antibody (panels d,h,l,p). Scale bar: 10 μm.



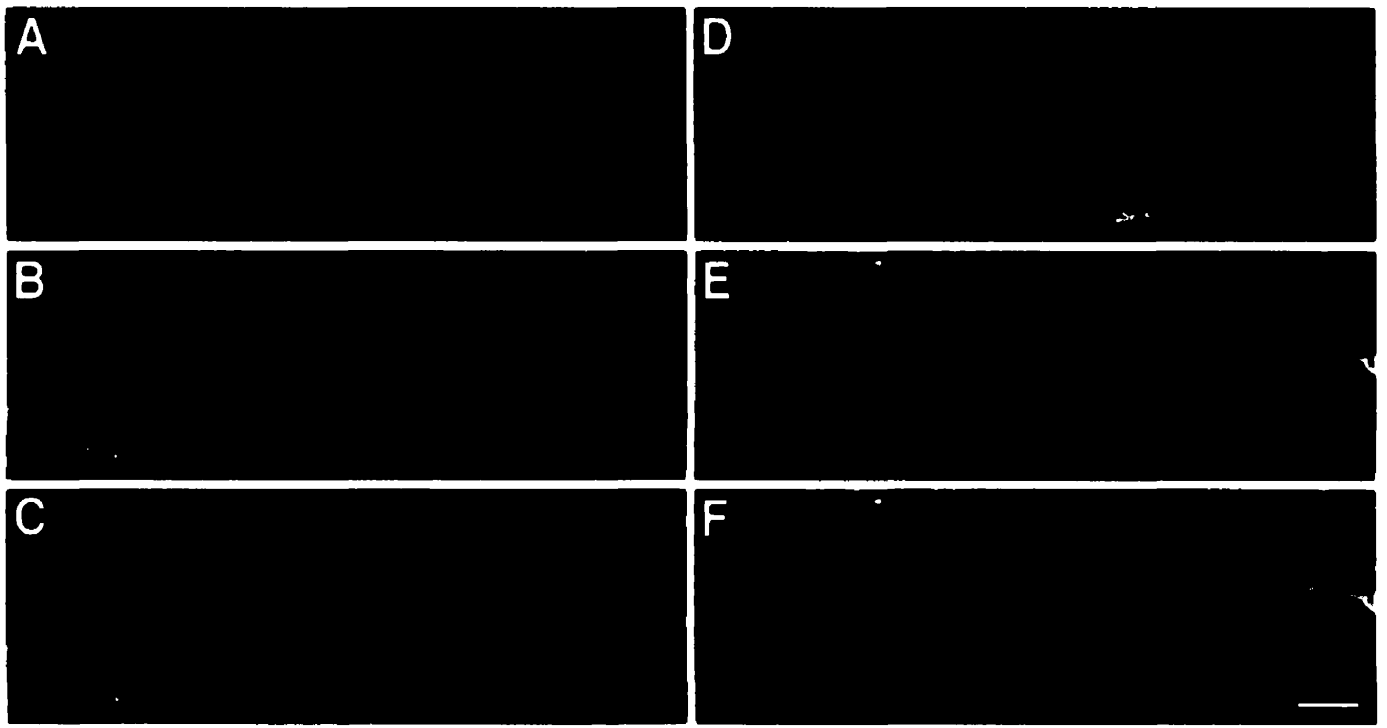
Manuscript Figure 2.

Figure 3. PMP22^{wtVSV} is incorporated into myelin *in vivo*. The sciatic nerve of P10 Sprague-Dawly rats was injected with 1-2 µl PMP22*wtVSV* recombinant adenovirus, excised five days later and subject to immunohistochemical analysis. Nerves were double labelled using a rabbit polyclonal anti-VSV G-protein antiserum conjugated to a Cy3 conjugated anti-rabbit immunoglobulin secondary antibody (panels a,d,g,j), and mouse monoclonal anti-MBP antibody conjugated to a FITC-conjugated anti-mouse immunoglobulin secondary antibody (panels b,e,h,k)(overlay in panels c,f,i,l, respectively). While some infected fibers demonstrate a distribution of newly synthesized PMP22^{wtVSV} around a negatively-stained oval structure as well as in the adaxonal Schwann cell cytoplasm adjacent to myelin (panels a-f), other fibers demonstrate a strong colocalization of MBP, a marker for compact myelin, and PMP22^{wtVSV}(panels g-l). Scale bar: 12 µm.



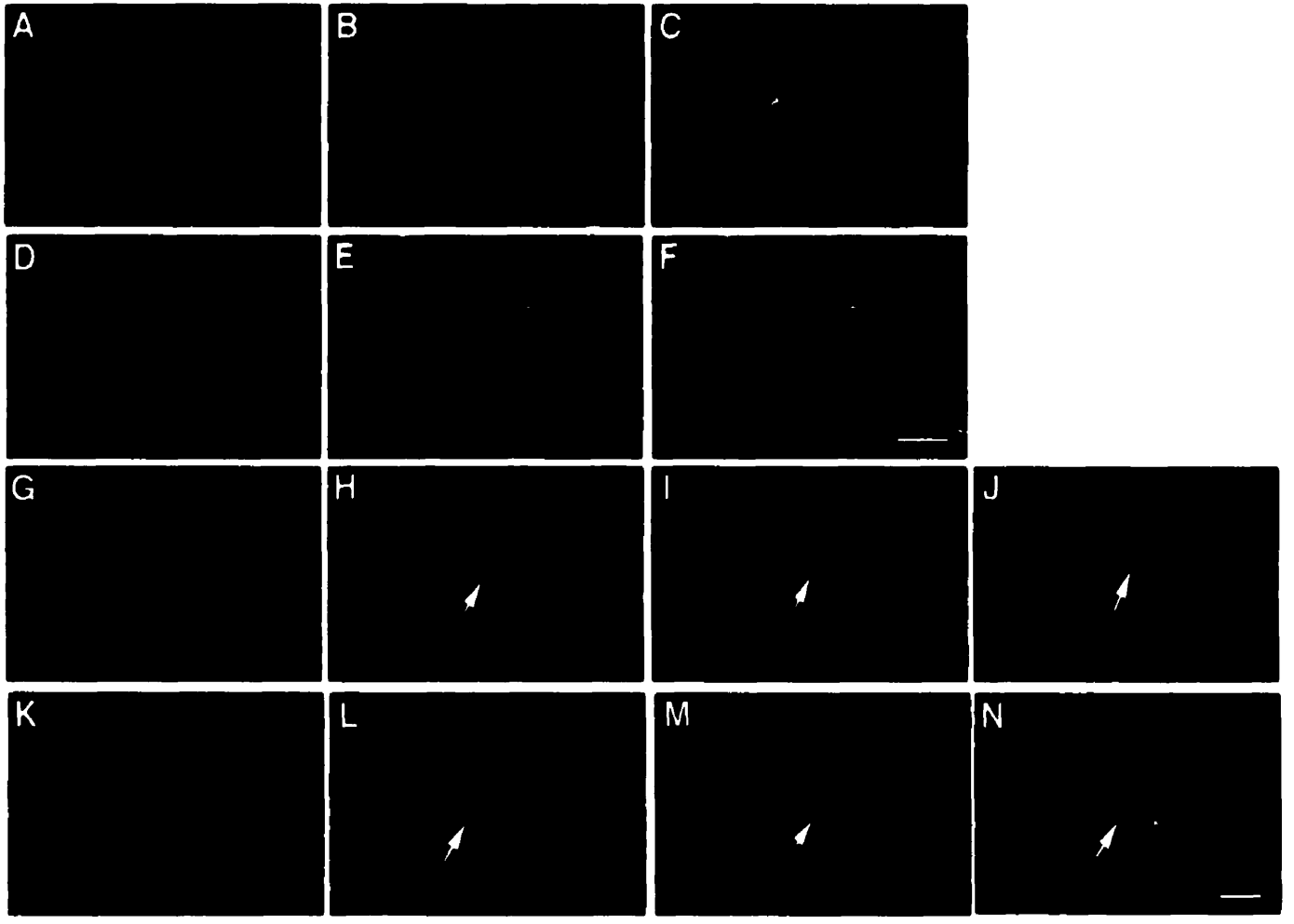
Manuscript Figure 3.

Figure 4. Epitope-tagged PMP22^{TrVSV} and PMP22$^{Tr\text{-}JVSV}$ are not incorporated into myelin *in vivo* . The sciatic nerve of P10 Sprague-Dawley rats was injected with 1-2 µl PMP22*Tr*VSV or PMP22*Tr-JVSV* recombinant adenovirus, excised five days later and subject to immunohistochemistry and confocal microscopy. Frozen sections were double labelled using a rabbit polyclonal anti-VSV G-protein antiserum conjugated to a Cy3 conjugated anti-rabbit secondary antibody (panels a,d), and a mouse monoclonal anti-MBP primary antibody conjugated to FITC-conjugated anti-mouse immunoglobulin secondary antibody (panels b,e)(overlays shown in panels c,f). In myelinating Schwann cells, PMP22^{TrVSV}(panels a-c) and PMP22$^{Tr\text{-}JVSV}$ (panels d-f) demonstrate an antiparallel distribution, tapering from a negatively-stained oval structure, which does not colocalize with MBP. Scale Bar: 12 µm.



Manuscript Figure 4.

Figure 5. PMP22^{Tr} and PMP22$^{Tr\text{-}J}$ show strong colocalization with the resident ER protein; PDI. The sciatic nerve of P10 Sprague-Dawly rats was injected with 1-2 µl *Tr*VSV or *Tr-JVSV* recombinant adenovirus, excised five days later and subject to immunohistochemistry. Frozen sections were triple labelled using a rabbit polyclonal anti-VSV G-protein antiserum conjugated to a Cy3 conjugated anti-rabbit secondary antibody (panels a,d,g,k), a mouse monoclonal anti-PDI primary antibody conjugated to FITC-conjugated anti-mouse immunoglobulin secondary antibody (panels b,e,h,d)(overlays shown in panels c,f,j,n), and Hoerchst dye (panels i, m). Confocal images of PMP22^{TrVSV} (panels a-c) and PMP22$^{Tr\text{-}JVSV}$ (panels d-f) expressing, myelinating Schwann cells show strong colocalization of the mutants with PDI. Images of triple-labelled nerves, taken on a standard fluorescence microscope indicate that the negatively stained oval structures observed in PMP22^{TrVSV} (panels g-j) and PMP22$^{Tr\text{-}JVSV}$ (panels k-n) expressing fibers represent the nuclei of the Schwann cells. Scale bars: 12 µm



Manuscript Figure 5.

3. Summary and Conclusions

3.1. Summary

The goal of this thesis was to determine the intracellular trafficking of the *Trembler* and *Trembler-J* forms of PMP22 in a myelinating Schwann cell *in vivo* and to establish a model system for future studies of myelination. Devising an *in vivo* system was necessary to overcome the limitations of *in vitro* systems of studying mutant myelin proteins. Such *in vitro* systems lack axon-glial interaction, and other potential factors of the natural environment that can influence the cell biology of the Schwann cell. We developed a rapid system for introducing mutant myelin protein into adenovirus. Replication-defective recombinant adenoviruses expressing epitope-tagged wild-type, *Trembler* and *Trembler-J* PMP22 as well as the *lacZ* reporter gene were generated. A surgical protocol to microinject the recombinant adenoviruses into rat sciatic nerve was established. This provided a system to transduce Schwann cells *in vivo*. The *lacZ* reporter gene was used to assay the efficiency of Schwann cell transaction in infected nerves. Histological examination revealed that, although there were some unlabelled fibres showing Wallerian degeneration (most likely as a result of mechanical damage caused by the injection procedure), transduced Schwann cells maintained structural integrity and developed normal myelin sheaths (introduction, figure 1). Therefore, a replication-defective recombinant adenovirus approach has provided a robust system to deliver expression cassettes into myelinating Schwann cells *in vivo*. In this way, myelinating Schwann cells were transduced to express the epitope-tagged wild-type, *Trembler*, or *Trembler-J* forms of PMP22.

To determine whether the epitope-tagged protein could be incorporated into myelin, double-label immunofluorescence of nerves injected with the wild-type PMP22 expressing adenovirus using antibodies against the VSV epitope tag and myelin basic protein (MBP) was carried out. This revealed that newly synthesized epitope-tagged wild-type PMP22 is efficiently transported along the length of the myelinating Schwann cell where some of it is incorporated into myelin. In contrast to the epitope-tagged wild-type PMP22, no colocalization of the epitope-tagged *Trembler* or *Trembler-J* mutant PMP22

with MBP was observed. Therefore, the epitope-tagged PMP22 carrying the *Trembler* and *Trembler-J* mutations are not incorporated into the myelin sheath. Rather, epitope-tagged *Trembler* and *Trembler-J* were distributed in an antiparallel intracellular compartment around the nucleus extending bidirectionally in a tapering manner along the axon. The consistent colocalization of the epitope-tagged mutants with the ER resident protein PDI revealed that the newly synthesized *Trembler* and *Trembler-J* PMP22 is largely retained in the ER of myelinating Schwann cells. In sum, we have determined that in myelinating Schwann cells *in vivo*, newly synthesized epitope-tagged wild-type PMP22 is incorporated into myelin whereas the representative PMP22 mutants, *Trembler* and *Trembler-J*, are intracellularly retained in the ER. Furthermore, we have validated a model system to study mutant myelin proteins in Schwann cells *in vivo*.

3.2. Conclusion

3.2.1. The gain-of-function effects of trembler and trembler-j PMP22 occur in the Schwann cell ER

Previous studies on *Trembler*, *Trembler-J* and PMP22 knock-out mice have demonstrated that the *Trembler* and *Trembler-J* mutants do not simply act as a passive loss of one *PMP22* allele. Rather, *Trembler* and *Trembler-J* PMP22 appear to alter the Schwann cell through an active, but undetermined mechanism. The phenotypical discrepancies between heterozygous *Trembler*, *Trembler-J* and heterozygous PMP22 knockout mice emphasize the active influence of *Trembler* and *Trembler-J* PMP22 on the Schwann cell. (Adlkofer *et al.* 1997). The mere presence of the *Trembler* allele in compound heterozygote mice consistently results in dramatic hypomyelination in (*Tr*/+), (*Tr*/0), and (*Tr*/*Tr*) mice while age-matched homozygote (0/0) and heterozygote (0/+) knock-outs display only focal hypermyelination with no signs of hypomyelination (Adlkofer *et al.* 1997). This has verified that the *Trembler* allele dynamically effects the Schwann cell. The drastic differences between the homozygote *Trembler*, *Trembler-J*, and PMP22 knock-out mice support this 'gain-of-function' effect of *Trembler* and *Trembler-J* PMP22 on myelinating Schwann cells (Henry *et al.* 1983a,b, Adlkofer *et al.* 1995).

Furthermore, comparisons of the phenotypes of *Trembler* and *Trembler-J* homozygote mice (extensively outcrossed to minimize potential genetic background artifacts), indicate that *the effect of these two alleles is different* (Henry *et al.* 1983).

As we have shown, PMP22 carrying the *Trembler* or *Trembler-J* mutations is largely retained in the ER of myelinating Schwann cells. We conclude that the action of the two mutants, as well as the variance in action between the two mutants, occurs primarily within the ER of the Schwann cell. Therefore, if we consider the effect of the two mutants at the cellular level, there exist two possible explanations: (i) the *Trembler* and *Trembler-J* PMP22 mutants manifest at two different points within the Schwann cell ER or (ii) the *Trembler* and *Trembler-J* mutants manifest at the same point in the ER of the Schwann cell, but to a varying degree.

The next step is to determine how the *Trembler* and *Trembler-J* mutants alter the passage of PMP22 through the Schwann cell ER. The *Trembler* mutation results in a glycine to aspartic acid (G150D), non-conservative amino-acid substitution that introduces a charged amino-acid into the fourth putative transmembrane domain of PMP22 (Suter *et al.* 1992a). The *Trembler-J* mutation results in a leucine to proline (L16P) non-conservative amino-acid substitution in the first putative transmembrane domain of PMP22 (Suter *et al.* 1992b). By altering the primary amino-acid sequence, the *Trembler* and *Trembler-J* mutations could hinder the proper formation of the secondary, and tertiary structure of nascent PMP22 within the ER. Both of these mutations introduce amino-acids into putative transmembrane-spanning domains (Welcher *et al.* 1991) that could alter their insertion into the phospholipid bilayer. Is misfolding of *Trembler* and *Trembler-J* PMP22 the fundamental problem?

As a disease pathway, improper folding of a mutant protein is not a novel concept. Over forty human diseases have been classified as disorders in protein conformation that affect ER processing including cystic fibrosis, diabetes mellitus, Perlizaeus-Merzbacher disease and CMT syndrome (reviewed by Pahl *et al.* 1997, reviewed by Aridor *et al.* 1999). We will examine several steps of protein synthesis within the ER that might be

crucial in the pathological action of *Trembler* and *Trembler-J* PMP22: First, the proper folding of nascent polypeptides within the ER is assisted by chaperones. Second, the ER promotes the degradation of aberrant ER proteins through an ER-associated degradation pathway (ERAD). Third, the accumulation of protein in the ER gives rise to an ER-overload response and finally, abnormal folding of protein within the ER results in impaired transport vesicle formation. The synthesis, folding and transport of PMP22 through the ER is almost entirely uncharacterized. Therefore, the remaining discussion will examine the aforementioned ER-responses to misfolded proteins. As nature often works in trends, we will try to relate these to PMP22

3.2.2. Do Chaperones aid in the folding and synthesis of proteins within the ER?

Membrane-bound or soluble proteins destined for intracellular organelles or the cell membrane are produced in the ER. To insure that nascent polypeptides attain their proper conformation, the ER provides a selective environment for complex protein folding. ER 'quality control mechanisms' minimize the export of partially folded protein intermediates or misfolded proteins from the ER. Thus, nascent polypeptides are introduced into the ER where the proper oxidative environment, the mechanisms for N-linked gylycosylation, and folding factors and chaperones assist in proper folding (reviewed by Stevens *et al.* 1999). It is likely that ER chaperones assist the proper folding and integration of nascent PMP22 into the membrane. Indeed, we have evidence that PMP22 interacts with one of these proteins, calnexin, *in vitro* and *in vivo* (Dickson *et al.* 1999).

Chaperones are molecules that bind to unfolded or misfolded proteins and in this way facilitate folding and prevent protein aggregation (Seckler *et al.* 1992). There are several classes of ER chaperones in the eukaryotic cell (reviewed in Zapun *et al.* 1999). Bip, a member of the Hsp70 family of proteins, is the most abundant ER protein and binds misfolded or unassembled proteins. Although its function is little understood, the glycoprotein Grp94 binds a number of unfolded polypeptides and is transcriptionally induced by stress to the ER. The lectin-like proteins, calnexin and calreticulin, bind to mono-glycosylated N-linked high mannose oligosaccarides on N-glycoproteins assisting

their folding as well as retaining them in the ER via the calnexin cycle (reviewed by Chevet *et al.* 1999). The protein disulphide isomerase (PDI) family of proteins assist in protein folding by catalyzing the formation and rearrangement of disulphide bonds. Reflecting the complexity of disulphide bond formation, a family of PDI-like proteins exists. Erp57 is one such protein that associates with calnexin and calreticulin to help in formation of glycoproteins. Families of peptidyl prolyl isomerases catalyze the isomerization of peptidyl prolyl bonds. Thus, the formation of PMP22 is likely scrutinized by more chaperones than calnexin.

Studying the associations of PMP22 with ER chaperones will be required to understand the cellular responses elicited by *Trembler* and *Trembler-J* PMP22. The retention of PMP22 within the ER provides a strong indication that misfolding of *Trembler* and *Trembler-J* PMP22 in the ER could give rise to their effect on the Schwann cell. A second line of evidence supporting misfolding of *Trembler* and *Trembler-J* PMP22 is the association of PMP22 degradation with ERAD and proteasome degradation.

3.2.3. ER-Associated Protein Degradation of Trembler and Trembler-J PMP22?

One pathway by which the cell prevents aberrant proteins from progressing past the ER is to target the proteins to the cytoplasmic ubiquitin-proteasome degratative pathway. This retrograde transport of aberrant proteins for cytoplasmic degradation is known as the endoplasmic reticulum associated degradation pathway (ERAD, Brodsky *et al.* 1999). Mutant Cystic Fibrosis transmembrane conductance receptor (CFTR) is degraded through the ERAD pathway (Ward *et al*, 1995). We will examine the degradation of mutant CFTR as a potential model for ER-associated degradation of PMP22. Like PMP22, CFTR is an N-glycosylated multispanning-transmembrane protein that associates with calnexin (Pind *et al.* 1994). Furthermore, PMP22 has been associated with ERAD *in vitro* (Notterpek *et al.* 1999).

The ubiquitin-proteasome pathway is a complex, controlled pathway of intracellular protein degradation that plays a role in many cellular processes. Historically, it

was thought to influence only cytoplasmic and nuclear proteins. However, recent findings have indicated that it plays an important role in the ER quality control pathway for membrane-anchored and secretory proteins. The process of proteasome mediated degradation involves the covalent attachment of multiple ubiquitin molecules to the target protein by a cascade of ubiquitin conjugating enzymes (reviewed by Ciechanover, 1994). The polyubiquitin tail acts as a recognition signal for the 26S proteasome thus targeting the protein for proteosomal degradation. The 26S proteasome complex is composed of a 20S catalytic core and a 19S ubiquitin–binding cap complex (Peters *et al.* 1994). Proteasomes and membrane embedded ubiquitin transferases have been associated with the membrane of the ER (Rivett *et al.* 1993, Sommer *et al.* 1993).

Newly synthesized membrane-anchored proteins are introduced into the phospholipid bilayer of the ER through the translocon, an evolutionarily conserved heterotrimeric protein translocation channel, Sec61 (reviewed by Rapoport *et al.* 1996). For membrane protein translocation, it is hypothesized that the Sec61 complex opens to allow the lateral exit of the transmembrane sequence of the nascent polypeptide into the lipid bilayer (Singer *et al.* 1987). The manner in which multispanning membrane proteins exit the translocon is unclear (reviewed by Matlack *et al.* 1998). Nonetheless, it seems that this is the first step at which nascent polypeptides are scrutinized for proper folding. Both nontranslocating and newly targeted Sec61 translocons are sealed lumenally by the primary peptide-binding chaperone BiP (Hamman *et al.* 1998). Functional mutant analyses have linked the translocon and BiP to retrograde transport for ER degradation (Plemper *et al.* 1997). It can be predicted that nascent CFTR and PMP22 are introduced into the phospholipid bilayer through the translocon. Similarly, it is possible that the translocon could be involved in the retrograde transport of PMP22 to ERAD.

ERAD has been associated with Cystic Fibrosis. The Cystic Fibrosis transmembrane conductance receptor (CFTR) is a core-glycosylated, multispanning membrane protein that serves as a secretory Cl^- channel (Riordan *et al.* 1989). 90% of patients with cystic fibrosis carry a deltaF508 mutation on at least one CFTR allele (Collins *et al.* 1992). Like PMP22, most post-translationally glycosylated CFTR remains in

a pre-Golgi compartment where it is degraded (Cheng *et al.* 1990). Furthermore, almost all immature glycosylated CFTR remains associated with the ER chaperone calnexin and cytoplasmic Hsp70 (Pind *et al.* 1994, Yang *et al.* 1993). Ward *et al.* have shown that wild-type and mutant CFTR is degraded by the ubiquitin-proteasome pathway (Ward *et al.* 1995). Degradation of wild-type and mutant CFTR can be blocked by specific inhibitors of the 20S catalytic subunit of the 26S proteasome. This leads to the accumulation of polyubiquitinated forms of CFTR. Polyubiquitinated forms of CFTR are detectable *in vivo*. Furthermore, preventing polyubiquitination effectively inhibits the degradation of CFTR suggesting that polyubiquitination is essential in this proteasome mediated degradative pathway. Jensen *et al* they proposed that a second degradation pathway worked in parallel to ERAD as 20S proteasome-inhibitors did not increase the expression of mature CFTR (Jensen *et al.* 1996). As CFTR and PMP22 are both N-glycosyated multispanning-transmembrane proteins that associate with calnexin, it is quite possible that newly synthesized *Trembler* and *Trembler-J* PMP22 are degraded in a similar manner. However, it is unclear how transmembrane proteins like CFTR or PMP22 could be expelled from the phospholipid bilayer for degradation via ERAD. The virally induced degradation of multi-histocompatibility class1 (MHC1) heavy chains can provide some insight as to how this might occur.

Membrane-spanning core-glycosylated class MHC1 heavy chains are targeted from the ER membrane to a proteasome-mediated degradative pathway by the human cytomegalovirus (HCMV, Wiertz *et al.* 1996a,b). Emmanuel *et al.* have shown that the HCMV membrane anchored viral protein US2 associates with newly synthesized MHC1 heavy molecule escorting it back to the cytoplasm where it is deglycosylated by cytoplasmic N-glycanase, and subsequently degraded by the 26S proteasome (Wiertz *et al.* 1996a). The association of pre-degradation intermediates with the sec61 translocation complex suggests that this pathway could represent a reversal of the protein translocation pathway, the lateral release of a membrane anchored protein from the lipid bilayer back into the lumen of the sec61 protein-conducting channel. However, it must be recognized that the observed degradation could be carried out by previously described ER membrane-associated proteasomes (Rivett *et al.* 1993). Wiertz *et al.* (1996a) were unable to detect

ubiquitinated intermediates of degradation which could be indicative of two things; (i) ubiquitinated intermediates are too transient to detect or (ii) the heavy chain is degraded through a ubiquitin-independent proteasome pathway (Jariel-Encontre *et al.* 1995). Although virally induced, the HCMV induced MHC1 proteasome-degradation demonstrates a potential mechanism by which membrane proteins such as the CFTR or PMP22 re-enter the sec61 channel from the ER lipid bilayer for ERAD export. Furthermore, it suggests that cytoplasmic deglycosylation and proteasome mediated degradation of ER membrane-spanning proteins in an ubiquitin-independent manner cannot be ruled out.

Notterpek *et al.* (1999) have reported that in cultured cells expressing PMP22, inhibition of the proteasome pathway leads to the accumulation of PMP22 in perinuclear cytoplasmic structures which they propose are aggresomes. Observing that PMP22 overexpressed in Schwann cells accumulates in the perinuclear region, they further propose that aggresomes are part of the CMT1a disease pathway. The development of aggresomes in mammalian cells is thought to be a cellular response that restricts the damage caused by intracellular protein aggregation resulting from saturation of the ERAD-proteasome pathway (Johnston *et al.* 1998). Johnston *et al.* have characterized the formation of 'aggresomes' which they define as "*pericentriolar membrane-free cytoplasmic inclusions containing misfolded, ubiquitinated protein ensheathed in a case of intermediate filaments.*" Aggresomes were discovered as aggregates of misfolded polyubiquitinated CFTR and presenelin-1, a 43 kDa integral membrane protein associated with early onset familial Alzheimer's disease (Doan *et al.* 1996, Cruts *et al.* 1998). Therefore, the discovery of PMP22 induced aggresome formation *in vitro* indicates that PMP22 can be degraded through the ERAD. Aggresomes appear as electron-dense aggregates *in vitro* (Johnston *et al.* 1998). It is tempting to speculate that the electron-dense cytoplasmic debris reported in Schwann cells of *Trembler* and *Trembler-J* mice corresponds to aggresomes *in vivo* (Ayers *et al.* 1973, Low, 1976, Henry *et al.* 1983). If so, the *Trembler* and *Trembler-J* mice could provide direct evidence of the formation of aggresomes in a diseased state *in vivo*. At this point, it is difficult to predict the effect that proteasome mediated degradation and aggresome formation could exert on a myelinating Schwann cell.

3.2.4. Trembler and Trembler-J might elicit the Unfolded Protein Response of the Endoplasmic Reticulum

Misfolded *Trembler* and *Trembler-J* PMP22 in the ER of a Schwann cell could induce an ER-unfolded protein response (UPR). This could influence the expression of protein and lipid synthesis within the Schwann cell that could lead to altered myelin biogenesis. The ER is becoming increasingly accepted as dynamic structure that adjusts to the demands of the cell by altering nuclear transcription. The presence of misfolded proteins in the ER results in the activation of a signaling response from the ER to the nucleus leading to increased transcription of genes encoding ER chaperones and enzymes responsible for phospholipid biosynthesis (McMillan *et al.* 1994). The UPR is hypothesized to play a function in general ER homeostasis as well as the cellular response to abnormal protein folding (Cox *et al.* 1997). The mechanism of UPR signaling in yeast *saccharomyces cerevisaie* has provided a model of UPR signaling and will be summarized here (reviewed by Pahl *et al.* 1999).

Under a variety of conditions that compromise proper folding in the ER, the UPR is induced in yeast (Shamu *et al.* 1994). The transmembrane kinase Ire1p contains a cytoplasmic endoribonuclease domain and is localized to the ER membrane or the inner nuclear membrane (Mori *et al.* 1993). In the presence of misfolded proteins Ire1p appears to undergo trans-autophosporylation following oligomerization (Welihinda *et al.* 1996) at which point the cytoplasmic endoribonuclease becomes activated (Sidrauski *et al.* 1997). The endoribonuclease activity of Ire1p splices the constitutively expressed transcription factor HAC1 mRNA. The tRNA ligase RLG1 ligates the spliced HAC1 activating it (Kawahara *et al.* 1997, Sidrauski *et al.* 1996). Active HAC1 (Cox *et al.* 1996, Kawahara *et al.* 1997) translocates to the nucleus where it binds a 22-bp UPR element that is conserved in the five yeast UPR target genes and is essential for their induction by ER-stress (Mori *et al.* 1992). To recapitulate, an ER-kinase/endoribonuclease senses unfolded protein in the ER, splices an untranslated mRNA which is then translated becoming a transcription factor that induces the expression of ER chaperones (reviewed by Chapman *et al.* 1996).

As in yeast, eukaryotic cells that are subject to conditions that promote improper folding in the ER display a robust UPR. This results in the increased expression of the ER chaperones; BiP(GRP78), PDI(Erp59), GRP170, GRP94, ERP72, and GRP58 (reviewed by Chapman *et al.* 1997). In addition, the UPR induces a repression in protein synthesis by the cell (Wong *et al.* 1993). However, in contrast to yeast, there is no single ER-stress response element responsible for the UPR in eukaryotic cells. Rather, a number of functionally redundant elements might carry out a similar signaling function (Wooden *et al.* 1999). Recently, the human homologue to Ire1p was cloned and has similar functional properties to yeast Ire1p (Tirasophon *et al.* 1998). A eukaryotic cell gene encoding a type 1 transmembrane ER-resident protein (PERK) has been cloned. PERK resembles Ire1p in the lumenal domain, and has a cytoplasmic protein-kinase domain. ER stress increases PERK's kinase activity, at which point it phoshporylates eukaryotic initiation factor-2a (eIF2a) (Harding *et al.* 1999). Given that the eukaryotic cell is capable of undergoing an unfolded protein response, it is possible that in response to misfolded *Trembler* or *Trembler-J* PMP22 within the ER, a Schwann cell could elicit a UPR. In this scenario, this could have broad ramifications in terms myelin protein synthesis. Furthermore, the UPR may be connected to lipid biogenesis which has obvious implications for myelin formation (Cox *et al.* 1997).

Lipid biogenesis in a myelinating Schwann cell is a complex and developmentally regulated process (Siegal *et al.* 1994, and references therein). The UPR might alter lipid biogenesis in the Schwann cell. Inositol is a precursor to membrane phospholipids. In yeast, the transcriptional control of enzymes which regulate phospholipid biosynthesis are controlled by the inositol response element UAS^{ino}, based on levels of Inositol (Cox *et al.* 1997). The UPR and the Inositol response appear to be regulated in a coordinated fashion. The IRE1 gene was described as being essential for inositol pleiotrophy in yeast (Nikawa *et al.* 1992). Furthermore, mutants that are unable to mount a UPR are Inositol auxotrophs. As the ER is the major site of lipid synthesis and membrane production, it is understandable that an increase in ER protein production is associated with an increase in lipid biogenesis. The concentration of inositol and unfolded protein are controlled by

activation of the UPRE and UAS^{ino}. The points at which these two signaling pathways overlap has not been determined. However, activation of the UPR in myelinating Schwann cells in response to misfolded *Trembler* and *Trembler-J* PMP22 in the ER could alter the lipid biogenesis of this highly membranous cell. In support of this, there is evidence that the synthesis of several lipid species is abnormal in the peripheral nerves of *Trembler* mice (reviewed by Garbay *et al.* 1999).

3.2.5. Accumulation of Trembler and Trembler-J PMP22 in the ER could elicit the ER-overload response

Additionally, the accumulation of *Trembler* or *Trembler-J* PMP22 in the ER could elicit the ER overload response. The ER overload response constitutes a novel signaling pathway that appears to stem from the accumulation of integral membrane proteins in the ER. This novel pathway was proposed upon the observation of activation of the transcription factor NF-kB following the expression viral membrane proteins in the ER as well as ER retention of a number of native mammalian integral membrane proteins (Pahl *et al.* 1996, 1997, 1999). Indeed, the accumulation of membrane proteins in the ER seems to provide enough stimulus to induce the NF-kB response (reviewed by Pahl, 1999). NF-kB is a transcription factor that is released in response to viral and bacterial cell compromise, inflammatory cytokines, and irradiation. NF-kB exerts a broad array of functions primarily mediating immune and inflammatory responses (Baeuerle *et al.* 1994). Ca2+ efflux from the ER and reactive oxygen intermediates appear to be involved in the EOR signaling cascade (Pahl, 1996, letter). It has been proposed that the NF-kB response is evolutionary a broad cellular response to viral infection. In diseases that are associated with misfolding, the NF-kB pathway could be activated. In support of this concept, patients suffering from cystic fibrosis develop cytokine mediated pulmonary inflammation (Konstan *et al.* 1997). However, there is little evidence of cytokine mediated inflammation in the *Trembler* and *Trembler-J* nerves (Garbay *et al.* 1999, Martini *et al.* 1994). This indicates that if *Trembler* or *Trembler-J* PMP22 is capable of eliciting the EOR, it is not robust enough to promote severe cytokine mediated inflammation within the peripheral nerve. As the EOR is a novel pathway, it could invoke more subtle cellular responses that are not yet characterized.

3.2.6. PMP22 and Regulated protein export from the ER

There is evidence suggesting that the proper folding of integral membrane glycoproteins is a prerequisite for their export from the ER (Aridor *et al.* 1999). Therefore, misfolded *Trembler* and *Trembler-J* PMP22 could be retained in the ER as a result of abnormal transport vesicle formation. Protein export from the ER is an active process regulated by a number of proteins that constitute the coat protein complex (COP) II. Membrane-bound and soluble cargo proteins destined for ER export interact with Sar1 and Sec23/24 components of the COPII coat followed by interaction with Sec13/31 (Aridor *et al.* 1998, Kuehn *et al.* 1998). The interactions between cargo and coat complexes that selectively trigger the formation of transport vesicles have received increasing attention as there is evidence suggesting that the proper conformation of the cargo protein is required to trigger COP II vesicle formation (Aridor *et al.* 1998, Kuehn *et al.* 1998).

Aridor *et al.* (1999) studied the influence of VSV-G protein on the formation of COP II transport vesicles. The VSV-G^{TS} protein is a temperature-sensitive mutant of the VSV-G type I transmembrane surface glycoprotein. When expressed in eukaryotic cells at non-permissive temperatures, VSV-G^{TS} is improperly folded and thus retained in the ER. At the permissive temperature, VSV-G^{TS} is properly folded and transported out of the ER in a COPII dependant manner. Using this system, Aridor *et al.* (1999) demonstrated that misfolded VSV-G^{TS} leads to a reduction in the formation of COPII vesicle budding while proper folding of VSV-G^{TS} enhances budding. Observing that VSV-G interacts with the Sar1 and Sec23/24 components of the COPII machinery, they propose that both cargo and COPII machinery can modulate the formation of pre-budding intermediates involved in vesicle formation. In the light of these experiments, misfolded *Trembler* and *Trembler-J* PMP22, as well as other PMP22 mutants could be retained in the ER as a result of deficient COPII vesicle formation.

3.3. Future Directions: An optimistic Outlook.

It is likely that the proper synthesis of PMP22 in the ER is a highly scrutinized and carefully orchestrated process. However, very little is known about the passage of PMP22 through the ER. The identification of the *Trembler* and *Trembler-J* PMP22 mutants as *in vivo* ER-retention mutants opens many avenues of experimental inquiry that can be used to develop the understanding of general ER mechanisms and responses, as well as the pathogenic action of PMP22 in hereditary neuropathies. Determining the nature of interactions between PMP22 with ER chaperones will help to determine whether misfolded or overexpressed PMP22 can induce an unfolded-protein-response or an ER-overload response. In such a scenario, how could a PMP22 induced ER-response effect myelinating Schwann cells? Are wild-type and mutant PMP22 degraded through the ubiquitin-proteasome pathway? Does improper folding cause impaired vesicle formation? With the most common cause of CMT being a duplication in the PMP22 gene, it is possible that overexpression of PMP22 manifests as chronic ER-stress leading to a cellular response that is harmful to healthy myelin.

When Charcot, Marie and Tooth described the peroneal muscle atrophies one hundred and fourteen years ago, cell biology was an unexplored realm. Today, modern molecular tools to understand and manipulate the human cell are growing in number and becoming increasingly efficacious. Looking ahead then, there is hope that CMT patients will be offered some form of pharmaceutical intervention to improve their peripheral myelin. The results of this thesis imply that the search for such a pharmaceutical intervention might commence by understanding the passage of PMP22 through the Schwann cell ER.

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