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A Common Disease Mechanism for Hereditary Neuropathies Due to Point Mutations in the Peripheral Myelin Protein 22

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2. Zusammenfassung

Das Periphere Myelin Protein 22 (PMP22) ist ein kleines, hydrophobes Glykoprotein, das hauptsächlich von Schwann`schen Zellen exprimiert wird und einen wichtigen Bestandteil des kompakten Myelins des peripheren Nervensystem bildet. Funktionell ist PMP22 von entscheidender Bedeutung für die korrekte Myelinisierung während der Entwicklung peripherer Nerven, für die Homöostase der Myelinschicht und für die Erhaltung von Axonen. Aufgrund dieser Erkenntnisse wurde PMP22 eine Rolle als strukturelles Myelinprotein, sowie eine regulierende Funktion in der Proliferation und Differentiation der Schwann`schen Zellen zugesprochen.

Mit der Entdeckung einer Punktmutation im *pmp22* Gen der natürlich auftretenden dysmyelinisierenden Mausmutante *Trembler* (*Tr*) konnte ein ursächlicher Zusammenhang zwischen PMP22 und peripheren Neuropathien gezeigt werden. Tatsächlich geht aus aktuellen molekular-genetischen Studien hervor, dass Veränderungen wie Duplikationen, Deletionen und Punktmutationen des *PMP22* Gens für die häufigsten Formen vererbbarer motorischer und sensorischer Neuropathien (HMSN), wie die Charcot-Marie-Tooth'sche Krankheit (CMT1A), die vererbte Neuropathie mit Anfälligkeit zu Druckläsionen (HNPP) und ein Subtyp des Dejerine-Sottas-Syndroms verantwortlich sind.

Mit unseren Studien beabsichtigten wir den Beitrag einer veränderten Funktion oder Regulation von mutiertem PMP22 Protein zu möglichen Krankheitsmechanismen aufzuklären. Die *Tr* Mausmutante, die Veränderungen in der Physiologie der Schwann`schen Zelle und der Myelinisierung zeigt, diente als Modellorganismus zur Bestimmung der primären Konsequenzen des Gendefektes von PMP22 auf molekularer und zellulärer Ebene.
Eine immunhistochemische Untersuchung der peripheren Nerven von acht Monate alten, heterozygoten *Tr* Mausmutanten zeigte eine intrazelluläre Ansammlung von PMP22-immunoreaktivem Protein in Organellen der Schwann`schen Zellen, die dem Endoplasmatischen Reticulum (ER) oder

Golgi Apparat entsprachen. Dieses Phänomen ist wahrscheinlich auf einen veränderten Transport des mutierten PMP22 Proteins zurückzuführen. In Übereinstimmung mit dieser Hypothese wurde rekombinant exprimiertes Tr Protein im ER von transfektierten COS-7 und kultivierten Schwann`schen Zellen zurückgehalten, höchstwahrscheinlich aufgrund fehlerhafter Faltung und/oder beeinträchtigter Prozessierung. Darüber hinaus übte das Tr Protein in diesem *in vitro* System einen teilweisen dominant-negativen Effekt auf den Transport von gemeinsam exprimiertem Wildtyp PMP22 Protein vom ER zur Zelloberfläche aus.

In weiterführenden Experimenten erweiterten wir unsere Untersuchungen um eine Reihe von Mutationen des *PMP22* Gens, die in Patienten mit CMT1A, schwerer CMT1A, DSS und in einer vermuteten rezessiven Form der CMT1A gefunden worden waren.

Mit diesem Ansatz konnten wir damit bestätigen, dass unkorrekte Proteinfaltung und/oder beeinträchtigte Prozessierung eine Hauptursache für den Krankheitsmechanismus darstellt, der peripheren humanen Neuropathien zu Grunde liegt, die durch Punktmutationen im *PMP22* Gen verursacht werden.

Darüber hinaus konnten wir in unserem Zellkultursystem zeigen, dass die dominant vererbten mutanten PMP22 Proteine auch den Transport und die Prozessierung von gleichzeitig exprimiertem Wildtyp PMP22 stören.

3. Summary

Peripheral myelin protein 22 (PMP22) is a small, hydrophobic glycoprotein that is most prominently expressed by Schwann cells as a component of compact myelin of the peripheral nervous system (PNS). Its gene *pmp22* was independently identified as differentially regulated in various growth states of fibroblasts and after nerve injury.

Functionally, PMP22 is involved in correct myelination during development of peripheral nerves, the stability of myelin, and the maintenance of axons. While most of these functions relate to a role of PMP22 as a structural component of myelin, PMP22 has also been proposed as a regulator of Schwann cell proliferation and differentiation.

The discovery of a point mutation unveiled *pmp22* as the culprit gene for the naturally occurring dysmyelinating mouse mutant *Trembler (Tr)*. Recent progress in molecular genetics revealed that mutations affecting the *PMP22* gene including duplications, deletions and point mutations are responsible for the most common forms of hereditary motor and sensory neuropathies (HMSN) including Charcot-Marie-Tooth disease type 1A (CMT1A), Hereditary Neuropathy with Liability to Pressure Palsies (HNPP) and a subtype of Dejerine-Sottas Syndrome (DSS).

We intended to identify the contribution of an altered function or regulation of mutant PMP22 to possible disease mechanisms. The *Tr* mouse mutant showing alterations in Schwann cell physiology and myelination was chosen as a model organism to investigate the primary consequences of the gene defect on a molecular and cellular level.
An immuno-histochemical analysis of peripheral nerves of eight month-old heterozygous Tr mice showed an intracellular accumulation of PMP22-immunoreactive protein in compartments of Schwann cells reminiscent of the ER/Golgi apparatus. This phenomenon appeared to be a consequence of an altered transport of mutant PMP22 protein. In support of this hypothesis, recombinantly expressed Tr protein remains mainly retained in the ER

compartment of transfected COS-7 and cultured Schwann cells, most probably due to protein misfolding and/or impaired processing. In the same experimental approach, the Tr protein exhibits a partial dominant-negative effect on protein trafficking of wildtype PMP22 from the ER to the plasma membrane.

In a second round of investigations, we expanded our analysis to a wide variety of mutations that have been identified in human patients diagnosed for CMT1A, severe CMT1A, DSS and a presumed case of recessive CMT1A.
Using the same experimental paradigm we could identify improper protein folding and/or impaired processing as the major contributing factor to the disease mechanism underlying human peripheral neuropathies caused by point mutations in the *PMP22* gene. We could further confirm a dominant negative effect on the transport and processing of co-expressed wildtype PMP22 of those mutated PMP22 proteins that are dominantly inherited.

4. Introduction*

4.1. Structure and Expression of PMP22

Peripheral myelin protein 22 (PMP22) is a 160 amino acid integral membrane protein that is predominantly expressed by myelinating Schwann cells of the peripheral nervous system (PNS) (Snipes et al., 1992) where it is incorporated into compact myelin (Haney et al., 1996). PMP22 comprises 2-5 % of total myelin protein and consists of an 18 kDa polypeptide core which is predicted by molecular modeling to be arranged in several hydrophobic membrane-associated domains (D'Urso and Muller, 1997a; Suter and Snipes, 1995a) (Figure 1). An asparagine residue in the first extracellular domain is linked to approximately 4 kDa of carbohydrates and a sub-population of this glycosylation carries the L2/HNK-1 carbohydrate epitope (Hammer et al., 1993; Pareek et al., 1993; 1997; Snipes et al., 1993). This particular structure has been implicated in intercellular recognition and adhesion indicating that also PMP22 may be involved in similar processes (Schachner and Martini, 1995).

PMP22 was originally identified in two different systems: First as a PNS myelin protein that is downregulated after sciatic nerve injury in the distal nerve stump (Kitamura et al., 1976; Spreyer et al., 1991; Welcher et al., 1991), and secondly as a mRNA which is strongly up-regulated in growth-arrested NIH3T3 fibroblasts (called gas-3; Manfioletti et al., 1990; Schneider et al., 1988). Although by far the highest levels of PMP22 expression are observed in myelinating Schwann cells, PMP22 transcripts have also been detected in various other adult tissues, in particular in the intestine, lung and the heart (Patel et al., 1992; Taylor et al., 1995). In addition, motoneurons of most cranial nerve motor nuclei and of the ventral spinal cord express PMP22 (Parmantier et al., 1995). During development, PMP22 mRNA is found widespread in many different tissues of ectoderm-, endoderm- and mesoderm

*Parts of this introduction are published: "The Many Facets of the Peripheral Myelin Protein PMP22 in Myelination and Disease" Roland Naef and Ueli Suter in Microscopy Research and Technique 41 (1998), 359-371

origin, notably also in the central nervous system defining columnar and segmental domains in the rhombencephalon, mesencephalon and prosencephalon (Baechner et al., 1995; Parmantier et al., 1997).

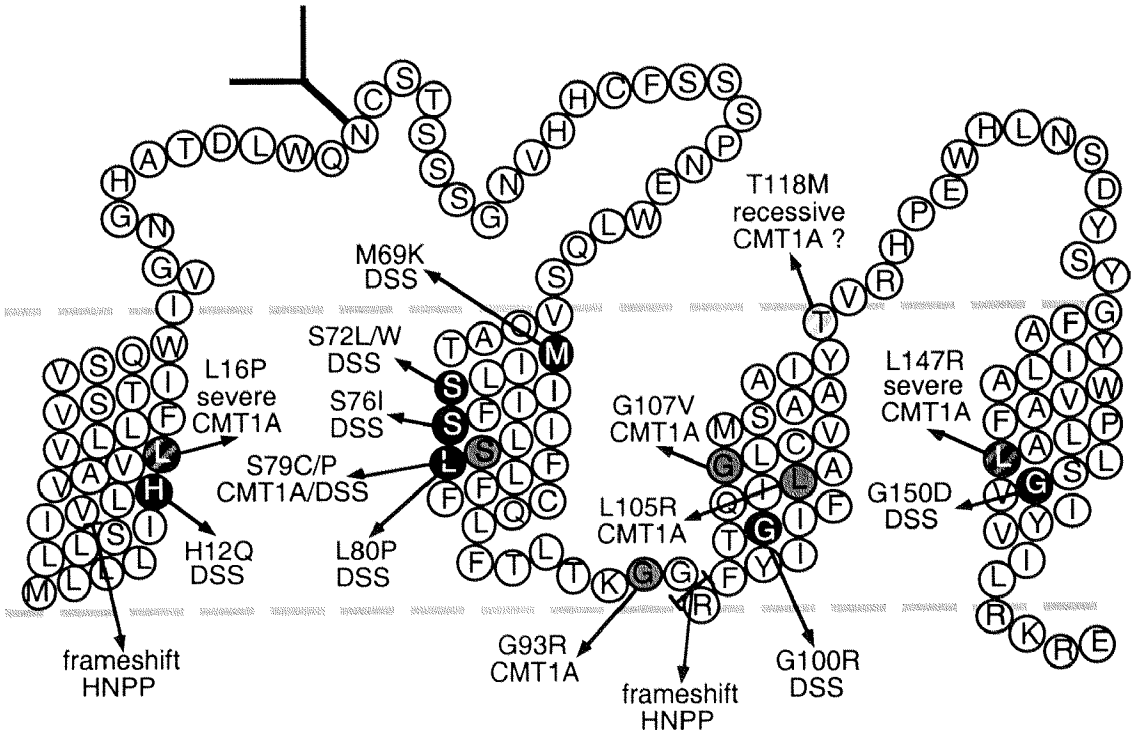

Figure 1: Disease mutations in a PMP22 structure model.

Disease causing mutations in the PMP22 protein are mainly clustered in the hydrophobic domains (for references, see text). Dark filled circles represent described DSS patients as used as synonym for the most severe phenotypes. Gray filled circles indicate CMT1A cases, and gray and black dashed circles represent intermediate phenotypes. A gray and white dashed circle marks the putative recessive mutation T118M. Note that frameshift mutations (I) lead to the comparatively mild disease HNPP. Broad gray dashed lines represent the borders of the plasma membrane with extracellular (e.g. intraperiod line in myelin) and the glycosylation site at position 41 (Y) on top and intracellular (e.g. major dense line in myelin) on the bottom.

4.2 Regulation of PMP22 Expression

Tissue-specific expression and regulation of PMP22 is complex. Two PMP22 gene promoters are linked to alternatively spliced untranslated exons (Bosse et al., 1994; Suter et al., 1994). Exon 1A-containing PMP22 transcripts, driven by the distal promoter (promoter 1), are found predominantly in myelinating Schwann cells and contribute most to the high levels of PMP22 in myelinated peripheral nerves while the exon 1B-containing PMP22 transcripts, regulated

by the proximal promoter (promoter 2), are present in all PMP22-expressing tissues. It is tempting to speculate that promoter 1 has been acquired specifically during evolution to allow high level expression in myelinating Schwann cells, a process which may have rendered an additional function to PMP22 as a structural component of compact myelin which may differ from the original (and likely retained) function of this protein. A similar evolutionary process has been suggested for some members of the crystallin gene family which are expressed as enzymes or stress proteins in many different tissues but have been subsequently recruited as structural components of the lens, most likely due to their favorable physico-chemical characteristics to accomplish this task (Wistow, 1993). However, PMP22 expression is also regulated posttranscriptionally. In growth-arrested NIH3T3 fibroblasts, the steady-state levels of PMP22 mRNA are increased by altered mRNA stability rather than enhanced transcription (Ciccarelli et al., 1990; Manfioletti et al., 1990). The molecular control of this regulatory mechanism remains to be clarified but it may also be functional in differentiated and quiescent myelinating Schwann cells. Interestingly, the level of exon 1A-containing PMP22 transcripts is greatly reduced in the hypomyelinated sciatic nerves of the *Trembler (Tr)* mouse (which is affected by a PMP22 point mutation, see below) while the amount of exon 1B-containing transcripts is not altered (Garbay et al., 1995). Whether this effect is due to a regulation at the transcriptional or posttranscriptional level remains to be determined but the results further support the proposed linkage between up-regulation of exon 1A-containing PMP22 transcripts and normal myelination.

In addition to PMP22 transcription and mRNA stability being tightly regulated, an another important level of the control of PMP22 expression is protein biosynthesis and translocation (Pareek et al., 1993; 1997). In contrast to the major PNS myelin protein P0, only a small portion of the newly synthesized PMP22 protein acquires complex glycosylation and reaches the Golgi apparatus of cultured Schwann cells. The vast majority of the PMP22 protein is retained in the endoplasmic reticulum (ER) and rapidly degraded. Surprisingly, this rapid intracellular turnover rate of PMP22 is not significantly altered in myelinating co-cultures of Schwann cells with sensory neurons or in

sciatic nerve explant cultures, although axonal contact promotes the translocation of PMP22 into myelin. An interesting parallel emerges with the biosynthesis of another well known and disease-associated polytopic membrane protein, the cystic fibrosis transmembrane conductance regulator, since most of this protein is also rapidly degraded within the cell due to aberrant folding (Ward and Kopito, 1994). It remains to be seen whether misfolding is indeed the reason for the abundant intracellular PMP22 degradation, possibly due to rate-limiting interactions with chaperones, or with other proteins that are transiently or stably associated with PMP22. Such a mechanism has been suggested as a potential explanation for why point mutations and increased gene-dosage may result in a similar cellular pathology in PLP/DM20 and PMP22 mutants by potentially increasing the amount of misfolded protein above a critical threshold level (Jung et al., 1996; Suter and Snipes, 1995a). Alternatively, it cannot be excluded that PMP22 acts as a chaperone-like protein with an intracellular function itself, or that oligomerization is involved in affecting the intracellular transport (potentially aberrant oligomeric PMP22 has been observed in rats with increased PMP22 gene dosage; Sereda et al., 1996). Interestingly, the caboxyterminal peptide of PMP22 (-LRKRE; Patel et al., 1992) resembles the consensus sequence of ER-resident membrane proteins with a type 1 topology (-K(X)KXX; Nilson and Warren, 1994). These basic amino acids are thought to be involved in a retrieval signal that is required to return lost ER proteins from the Golgi apparatus. To test the functionality of the PMP22-derived motif for ER retention, we have added these amino acids to the type 1 transmembrane protein IL2 receptor-alpha (IL2R alpha). Indeed, in contrast to the parent IL2R alpha which was efficiently transported to the plasma membrane of transfected COS-7 cells, the chimaeric IL2R alpha/PMP22 protein was largely retained in the endoplasmic reticulum and only minor amounts were able to reach the plasma membrane (Figure 2). Although little is known about the determinants of ER retention of polytopic membrane proteins, these data suggest that partial ER retention and/or retrieval may contribute to the observed regulation of PMP22 biosynthesis.

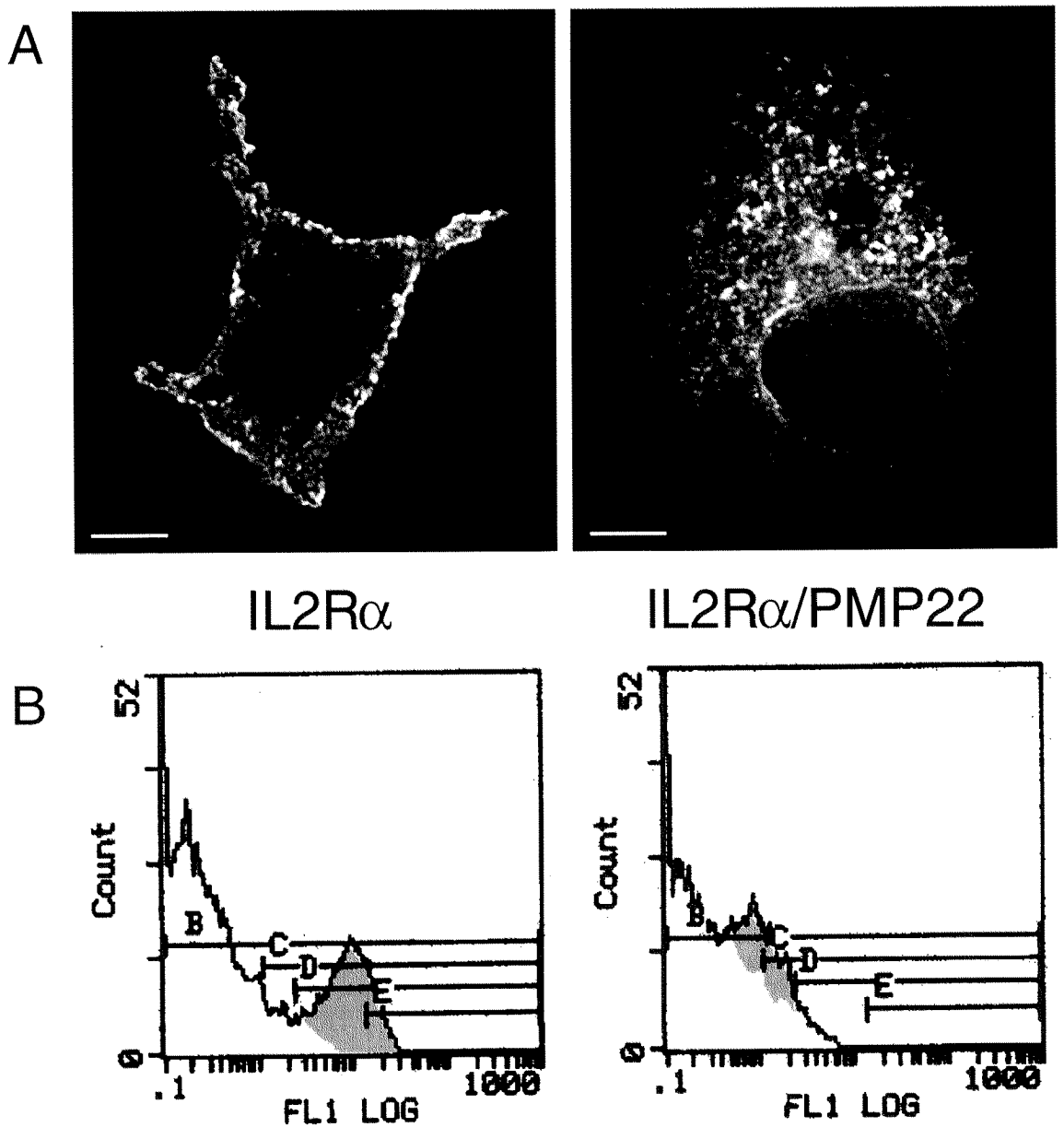

Figure 2: The C-terminal domain of PMP22 can act as a leaky ER-retention and/or retrieval signal.

COS-7 cells were transfected with the membrane protein IL2R alpha and a chimerical molecule consisting of the IL2R alpha fused to the last five amino acids (-LRKRE) of PMP22 (IL2R alpha/PMP22). Transfected cells were stained with monoclonal anti-IL2R alpha antibodies. A, wildtype IL2R alpha is readily transported to the plasma membrane while the chimera is mainly retained intracellularly (0,4 μm confocal optical sections are shown and the bar represents 10 μm).

B, FACS scan of live-stained transfected COS-7 cells indicates a transfection rate of 10-15% (shaded area) for both molecules. IL2R alpha is transported to the plasma membrane and stains brightly on the surface (left panel) as shown by a10-100 fold increase in fluoresence on the x-axis, while IL2R alpha/PMP22 is only weakly detectable (right panel). Quantitative analysis reveals that approximately 90-95% of the IL2R alpha/PMP22 protein is retained intracellularly (data not shown).

4.3 The PMP22/EMP/MP20 Gene Family

PMP22 belongs to small family of highly related proteins including the epithelial membrane proteins (EMP)-1, -2 and -3 which share 33 to 43% amino acid identities and similar genomic organizations (Ben-Porath and Benvenisty, 1996; Bolin et al., 1997; Lobsiger et al., 1996; Taylor and Suter, 1996). Most notably, the hydrophobic, potentially membrane-associated regions (including most of the disease-associated variants in PMP22) are highly conserved and the consensus sequence for N-linked glycosylation is positioned similar in all four proteins suggesting important functional and/or structural roles for these motifs (Lobsiger et al., 1996; Taylor and Suter, 1996). EMP-1 has been found by several laboratories in different tissues including high expression in epithelial cells of the gastric pit and the isthmus of gastric glands in the rat (Taylor et al., 1995), in a sub-population of immature B cells in human (clone called B4B; Ruegg et al., 1996) and in squamous epithelia in rabbits (clone called CL20; Marvin et al., 1995). In each of these systems, a function of EMP-1 in the regulation of cellular proliferation and differentiation was suggested. This hypothesis is further supported by the finding that EMP-1 is highly up-regulated in an invasive human mammary carcinoma cell line if compared to the non-invasive parental line (clone called PAP; Schiemann et al., 1997). Furthermore, EMP-1 is strongly expressed in c-myc-induced mouse brain tumor cells but not in normal brain tissue (clone called TMP; Ben-Porath and Benvenisty, 1996). Whether EMP-1 may have a growth-promoting function or its dramatically up-regulated expression is only a bystander effect remains to be determined.

EMP-2 is much less characterized and Northern blot analysis on human tissues revealed widespread expression, in particular in fetal lung and kidney as well as the adult ovary, heart, lung and intestine (Taylor and Suter, 1996). EMP-3 is also quite ubiquitously expressed but it is best characterized in the hematopoietic and the nervous system (called HNMP-1; Bolin et al., 1997). Specifically, various human hematopoietic and lymphoid lineages as well as adult mouse spleen and thymus express EMP-3. In the mouse nervous system, EMP-3 is expressed by Schwann cells, dorsal root ganglia sensory

neurons and motoneurons in the spinal cord. Immuno-histochemical analysis suggests that EMP-3 is axon-associated in the spinal cord fiber tracts and in the sciatic nerve. Furthermore, EMP-3 is up-regulated after peripheral nerve injury as well as in the hypomyelinated *Trembler-J (Tr-J)* mouse that carries a PMP22 point mutation (see below). It was concluded that EMP-3 might play a role in axon-Schwann cell interactions (Bolin et al., 1997). Based on the amino-acid identity patterns of PMP22 and the EMP polypeptides, together with the relationship of their respective gene structures, the lens-specific membrane protein 20 (MP20) was identified as an additional distant member of the same gene family (Lobsiger et al., 1996; Taylor et al., 1995). This hydrophobic protein with several potentially membrane-associated domains is prominently expressed in differentiating and mature lens fiber cells. It is likely to serve as a structural component in this system with an additional potential role in signal transduction (Galvan et al., 1989; Kumar et al., 1993; Mulders et al., 1988).

PMP22 can be classified further as a member of a large superfamily of potential tetraspan proteins that are associated with myelin but share no significant amino-acid identities with the PMP22/EMP/MP20 proteins. This group includes members of the PLP/DM20 family (Griffith et al., 1998; Gow et al., 1997a), the plasmolipin/MAL gene family (Fischer and Sapirstein, 1994; Gillen et al., 1996; Kim et al., 1995; Magyar et al., 1997; Schaeren-Wiemers et al., 1995) and the oligodendrocyte-specific protein (OSP) which may be distantly related to PMP22 (Bronstein et al., 1996). However, despite large experimental efforts and a multitude of information gathered on expression and regulation, the molecular function of most of these proteins in glial development and myelination has remained largely elusive. In the case of PMP22, it was mainly through spontaneous mouse mutants, the advent of modern human genetics and major technological advances in mammalian genetics (e.g. the generation of transgenic mice and rats) that we have learned about the crucial importance of proper PMP22 expression for the formation and maintenance of PNS myelin.

4.4 Genetics of PMP22 and its Involvement in Peripheral Neuropathies

Shortly after the characterization of PMP22 as a myelin gene, it was discovered to be affected by point mutations in the *Tr* (Suter et al., 1992a) and its allelic variant, the *Tr-J* mouse (Suter et al., 1992b). Soon thereafter, the most common forms of demyelinating hereditary motor and sensory neuropathies (Dyck et al., 1993; Gabreels-Festen et al., 1993; Harding, 1995), in particular Charcot-Marie-Tooth disease type 1A (CMT1A, HMSN IA), a genetic subtype of Dejerine-Sottas syndrome (DSS, HMSN III) and hereditary neuropathy with liability to pressure palsies (HNPP) were found to be associated with mutations affecting the *PMP22* gene (Suter and Snipes, 1995a, b).

CMT1A is an autosomal dominant disease with highly variable manifestations. It presents usually in the second to third decade of life as loss of muscle strength, beginning distally and slowly progressing to involve many muscle groups, with less significant involvement of sensory modalities (Birouk et al., 1997; Lupski et al., 1991a; Thomas et al., 1997). Marked decreases in nerve conduction velocities (NCV) are a hallmark of CMT1. Histologically, demyelination of peripheral nerves is observed which is associated with Schwann cell hypertrophy leading to the characteristic formation of Schwann cell onion bulb structures and often increased collagen depositions. Genetically, the CMT1A locus has been linked to chromosome 17p11.2 and identified as an intrachromosomal duplication of approximately 1.5 megabases in 70% of clinically diagnosed CMT patients (Lupski et al., 1991b; Nelis et al., 1996; Raeymaekers et al., 1991, Wise et al., 1996). Increased gene-dosage was initially proposed as a mechanism for CMT1A based on the observation of abnormal NCV in patients with segmental trisomies in this region (Chance et al., 1992; Lupski et al., 1992). The mapping of the intact *PMP22* gene within the duplicated region on chromosome 17, in conjunction with the finding of PMP22 mutations in *Tr* and *Tr-J* mice, identified *PMP22* as the prime candidate to cause the disease (Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992a, Warner et al., 1996).

This hypothesis was further supported by the finding of several *PMP22* point mutations in rare but severe cases of CMT1A (Gabreels-Festen et al., 1995; Marrosu et al., 1997; Navon et al., 1996 ; Ohnishi, 1995; Roa et al., 1993a; Valentijn et al., 1992b; Figure 1) and a subgroup of patients originally diagnosed with the congenital peripheral neuropathy DSS (Bort et al., 1997; Ionasescu et al., 1995; 1996; 1997; Roa et al., 1993b; Tyson et al., 1997; Valentijn et al., 1995; Figure 1). In addition, a potentially recessive *PMP22* point mutation has been described in a complex pedigree (Roa et al., 1993c) although the significance of this finding has been debated (Nelis et al., 1997). In general, the clinical phenotypes and the pathologies tend to be more severe in patients affected by *PMP22* point mutations compared to the effects of the intrachromsomal CMT1A-associated duplication (Gabreels-Festen et al., 1995; Tyson et al., 1997).

Deletions of a similar chromosomal segment that is duplicated in CMT1A (and spans the *PMP22* gene) are linked to the mild autosomal dominant hereditary neuropathy with liability to pressure palsies (HNPP; Chance et al., 1993). Consistent with a crucial function of PMP22 in this disorder, early frame-shifting mutations in this gene are also associated with HNPP (Nicholson et al., 1994; Young et al., 1997). Clinically, HNPP is characterized as a recurrent neuropathy which is usually precipitated by minor trauma to peripheral nerves and may progress to a more chronic disease with age (Cruz-Martinez et al., 1997; Windebank, 1993; Roa et al., 1995). Focal hypermyelinated structures with sausage-like appearance called tomacula on teased fiber preparations are the histological hallmarks of the disease but they may also be seen in other neuropathies (Thomas et al., 1994; Windebank, 1993).

Detailed structural analysis of the duplicated (CMT1A) or deleted (HNPP) locus revealed repetitive DNA elements (called REP) flanking the unstable region and potentially defining a recombination hotspot (Pentao et al., 1992; Reiter et al., 1997). Thus, it was suggested that the tandem duplication in CMT1A and the reciprocal deletion in HNPP are generated by an unequal crossing over between chromosome 17-homologues during meiosis (Chance et al., 1994). This process appears to have some preference for

spermatogenesis since the vast majority of *de novo* mutations are paternally derived (Lopes et al., 1997; Palau et al., 1993). Most interestingly, the recombination hot spot contains the remnants of an insect-derived mariner-like transposon element (Reiter et al., 1996). Although the specific transposase encoded by this locus is inactivated by mutations, it remains an intriguing hypothesis that other genome-encoded transposases that may be specifically active during spermatogenesis could mediate the proposed disease-causing crossover. Recent data suggest that this apparently sex-specific interchromosomal event leads exclusively to the CMT1A-associated duplication while other molecular mechanisms seem to account for the HNPP deletion (Lopes et al., 1997).

The knowledge gained from human genetics predicts PMP22 overexpression due to the heterozygous chromosomal duplication in CMT1A, and PMP22 underexpression due to the similar deletion in HNPP. Although initial PMP22 mRNA determinations on appropriate nerve biopsies have been controversial (Hanemann et al., 1994; Kamholz et al., 1994; Schenone et al., 1997; Yoshikawa et al., 1994), quantitative analysis of PMP22 expression on nerve biopsies by immunogold-labeling techniques revealed a definitive increase and decrease of PMP22 expression in CMT1A and HNPP, respectively (Vallat et al., 1996).

4.5 Lessons from Animal Models for PMP22-Related Neuropathies

Formal proof for the causative role of PMP22 in the described peripheral neuropathies and additional insight into the associated disease mechanisms was provided by the detailed analysis of naturally occurring and genetically engineered neurological animal mutants.

4.5.1 Genetically engineered PMP22-mutant animals

Mice with artificially reduced PMP22 expression

In order to examine the molecular function of PMP22 and to generate an animal model for PMP22 null mutations in human (e.g. HNPP), a PMP22-deficient mouse was generated by conventional gene "knock-out" techniques using homologous recombination in embryonic stem cells (Adlkofer et al., 1995). Mice which completely lack PMP22 are born and develop normally until the second week of life when walking difficulties due to progressive weakness of the hind limbs become obvious. Morphologically, a progressive peripheral neuropathy is observed which is characterized by a delayed onset of myelination (similar to observations in *Tr*, Ayers and Anderson, 1975), followed by the characteristic formation of paranodal and internodal hypermyelinated structures (tomacula) which are very prominent in approximately 3 weeks-old mice. At 10 weeks of age, however, tomacula are rarely observed but clear signs of demyelination and remyelination including thinly myelinated axons and Schwann cell onion bulb formations become abundant and are accompanied by very slow NCV (Adlkofer et al., 1995). Approximately one year-old homozygous mutants show some severely hypermyelinated and compressed axons, often associated with degenerating myelin as well as Schwann cell and basal lamina onion bulb formation. The neural phenotype is accompanied by a variable but usually strong muscular atrophy that is manifested by extensive type grouping of muscle fibers suggestive of denervation and axonal sprouting. No major pathological alterations in other tissues beside the PNS and their innervated tissues were detected, possibly due to compensatory effects by other members of the PMP22/EMP/MP20 family (Adlkofer et al., 1995).

Heterozygous "knock-out" mice mimic HNPP genetically and as expected, these animals show similar hallmarks as the human disease (Adlkofer et al., 1997b). There is a high variability between individuals but generally, increasing numbers of tomacula develop at a young age. These hypermyelination structures appear to be intrinsically unstable and tend to degenerate with aging, resulting in segmental demyelination and Schwann

cell onion bulb formation. The latter are classical signs of a CMT1-type demyelinating neuropathy and are likely to underlie the subtle electrophysiological abnormalities observed in these animals. These findings provide a plausible explanation for the sometimes overlapping clinical and pathological features of HNPP and CMT1 (Windebank, 1993; Roa et al., 1995). Furthermore, one might speculate that the increased instability of tomaculous myelin might also be involved in the transient symptoms experienced by HNPP patients after nerve trauma.

An alternative mouse model for HNPP has been generated by the expression of rat PMP22-antisense mRNA under the control of the Schwann cell-specific P0 promoter (Maycox et al., 1997). Despite a relatively marginal reduction of the endogenous PMP22 transcript level to approximately 85% in these mouse mutants, pathological signs of a neuropathy including hypermyelinated and hypomyelinated axons accompanied by electrophysiological abnormalities were observed. Interestingly, these mice show a pronounced high stepping gait that was not seen in other PMP22 mutant animals. This discrepancy may be attributed to the developmental regulation of the P0 promoter used to drive the transgene that may differ from the endogenous PMP22 gene. Alternatively, the observed defects may not be an exclusive consequence of the reduction of PMP22 expression but the expression of other genes may also be affected by the high levels of transgenic antisense mRNA. Furthermore, genetic background effects due to the different mouse strains used cannot be excluded.

Rodents with increased PMP22 gene dosage

Several approaches were chosen to generate appropriate models for the chromosomal duplication that is associated with CMT1A and contains the PMP22 gene. The most striking similarities to the human pathology were found in transgenic rats carrying three additional copies of a cosmid containing the mouse PMP22 gene controlled by its own regulatory elements (Sereda et al., 1996). These animals develop a CMT1-like neuropathy characterized by an unsteady gait, reduced NCV, and loss of muscle strength leading to deficits in muscle performance. Interestingly, quite a large variation

between individual transgenic littermates was observed comparable to the variable phenotypes within CMT1A families (Birouk et al., 1997; Kaku et al., 1993; Wise et al. 1993) and even in identical twins (Garcia et al., 1995) suggesting the involvement of stochastic factors, or environmental modulation of the disease. Morphological analysis revealed amyelinated large caliber axons intermingled with normally myelinated fibers while small caliber axons were often hypermyelinated. Strikingly, ventral roots were markedly more affected than dorsal roots suggesting more pronounced deficiencies of motor nerves. In addition, Schwann cell onion bulbs associated with too thinly myelinated axons indicative of demyelination and remyelination were frequently encountered. The gene-dosage effect of PMP22 became even more obvious when the transgene was bred to homozygosity. These rats were severely affected, retarded in growth and displayed almost complete paralysis of the hindlimbs. Morphologically, virtually no myelin was observed in the sciatic nerves of such animals with the exception of occasional membranous debris. PMP22 overexpression was detectable at the mRNA level in most mutant animals and correlated with clinical signs of disease while PMP22 protein levels were decreased similar to P0 and MBP, likely reflecting the morphologically observed hypomyelination.

Similar to the homozygous transgenic rats, mice carrying approximately 15 or 30 copies of the same PMP22-containing cosmid were largely amyelinated (Magyar et al., 1996). These animals display correct segregation of Schwann cells and large caliber axons into the 1:1 relationship but most of these units fail to myelinate resulting in very slow NCV. Schwann cell differentiation appears impaired as manifested by excessive proliferation and the maintained expression of proteins specific for non-myelinating Schwann cells. Only occasionally, some axons may escape the myelination block as indicated by normally myelinated small caliber axons in one year-old animals.

To complement the available set of PMP22-transgenic animals, another mouse strain that carries eight copies of a yeast artificial chromosome (YAC) containing the human *PMP22* gene displays an intermediate demyelinating phenotype (Huxley et al., 1996). The apparent dependence of the phenotype

in transgenic rodents on gene-copy number correlates well with the generally more severe phenotype of homozygous versus heterozygous CMT1A duplication patients although there is also considerable clinical variability (Kaku et al., 1993; Killian and Kloepfer, 1979; Lupski et al., 1991b; Le Guern et al., 1997; Sturtz et al., 1997)

4.5.2 Spontaneous PMP22 mutant mice: *Trembler and Trembler-J*

The autosomal dominant mouse mutants *Tr* and *Tr-J* are clinically affected by an unsteady gait, tremor and quadriparesis (Falconer, 1951; Henry et al., 1983) and have been crucial for the identification of PMP22 as the target gene in peripheral neuropathies (Suter et al., 1993). Furthermore, they provide an excellent model for a detailed analysis of myelin abnormalities and potential disease mechanisms due to PMP22 point mutations.

Tr mice carry a single guanine-to-adenine base transition resulting in the replacement of a glycine residue by aspartate in the last hydrophobic domain of PMP22. The mutants display severe hypomyelination already early in development with only a few myelinated fibers visible on peripheral nerve sections (Ayers and Anderson, 1975; 1976; Henry et al., 1983; Low, 1976a; 1976b), aberrant continuos Schwann cell proliferation (Perkins et al., 1981) and reduced NCV (Low and McLeod, 1975). Heterozygous *Tr* mice retain approximately 5-30% myelinated fibers whereas homozygous animals are virtually devoid of PNS myelin and develop a more severe phenotype which, however, does not affect their normal life span (Henry and Sidman, 1988). Older heterozygous *Tr* display empty basal lamina onion bulbs, most probably remnants of degenerated supernumerary Schwann cells and their processes (Low, 1977). Similar pathologies are characteristic for DSS (Ayers and Anderson, 1973) and indeed, the *Tr* point mutation has been also found in patients which have been diagnosed with DSS based on their severe clinical symptoms (Ionasescu et al., 1997). Structural abnormalities have been observed in the remaining Tr myelin by X-ray diffraction suggesting impaired compaction (Kirschner and Sidman, 1976).

At the molecular level, PMP22 mRNA levels (Bascles et al., 1992) and the compact myelin proteins P0 and MBP are strongly reduced in the hypomylinated *Tr* mouse (Garbay and Bonnet, 1992) and several components of the lipid metabolism are also affected (Boiron-Sargueil et al., 1995; Garbay and Cassagne, 1994; Heape et al., 1995; 1996). Furthermore, *Tr* reveals a severe defect in axon-Schwann cell interactions. Transplantation studies demonstrated that the main defect resides in the Schwann cell (Aguayo et al., 1977) and that the defective *Tr* Schwann cells lead to a decreased axonal diameter, increased neurofilament density, slow axonal transport and aberrant modulation of a kinase-phosphatase system acting on neurofilaments (de Waegh et al., 1992; Snipes and Suter, 1994). In addition, the stability of the axonal microtubule cytoskeleton is decreased and the composition and phosphorylation of microtubule-associated proteins, including tau, MAP 1A, and MAP 1B, are severely altered (Kirkpatrick and Brady, 1994). Nevertheless, a minor disease-contributing effect of motoneuron-expressed PMP22 cannot be completely excluded (Parmantier et al., 1995).

The *Tr-J* mutation (which has also been described in a human CMT1A family; Valentijn et al., 1992b) leads to a substitution of a leucine residue by a proline in the first putative membrane-associated domain of PMP22 (Suter et al., 1992b) resulting in a clinically similar but generally less severe pathology compared to *Tr* (Henry et al., 1983). Morphological examination revealed a dysmyelinating neuropathy including thinly myelinated axons and signs of demyelination and remyelination, aberrant Schwann cell proliferation and Schwann cell onion bulb formation (Henry et al., 1983; Notterpek et al., 1997). Subtle abnormalities in the compaction of myelin (Heath et al., 1991) and a failure of correct Schwann cell-axon interactions resulting in abnormal myelination and axonal loss in the dorsal roots have also been described (Robertson et al., 1997). Furthermore, the mutant *Tr-J* Schwann cells express an altered pattern of trophic factors (Friedman et al., 1996). As predicted from the observed myelin deficiencies, PMP22, P0 and MBP protein levels are markedly decreased in *Tr-J* nerves while MAG is only affected by altered glycosylation (Bartoszewics et al., 1996; Inuzuka et al., 1985; Notterpek et al., 1997).

5. Objectives

Since the identification of PMP22 as a major component of the compact myelin of peripheral nerves, a multitude of information concerning its expression and regulation in different adult and embryonic tissues and cell types were gathered. Most importantly, *PMP22* was identified as the culprit gene for the most common forms of hereditary motor and sensory neuropathies (HMSN). It became clear that PMP22 is involved in correct myelination during development of peripheral nerves, the stability of myelin, and the maintenance of axons. While most of these functions relate to a role of PMP22 as a structural component of myelin, PMP22 has also been proposed as a regulator of Schwann cell proliferation and differentiation. In contrast to all the proposed functions, little is known so far about the true molecular function of PMP22 and its potential interactions with known intra- and intercellular processes.

The aim of this study was to perform a detailed molecular and cellular analysis of the naturally occurring dysmyelinating *pmp22* point mutant *Tr* to identify such processes. Furthermore, we developed a cell culture based *in vitro* system to provide a better-controlled environment for the characterization of the observed effects in the *Tr* mouse. Second, we used our experimental paradigm to expand our study to a wide variety of point mutations identified in *PMP22* of patients suffering from different rare subtypes of HMSN to decipher a general disease mechanism for point mutated PMP22 proteins.

6. Results

6.1 Aberrant Protein Trafficking in *Trembler* Suggests a Disease Mechanism for Hereditary Human Peripheral Neuropathies

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6.1.1 Abstract

The naturally occurring mouse mutant *Trembler* (*Tr*) represents an animal model for inherited human neuropathies caused by point mutations affecting peripheral myelin protein 22 (PMP22). We describe the likely pathogenic cellular mechanism underlying the observed myelin deficiency. In *Tr/+* animals, PMP22 immunoreactivity was found not only in compact myelin but also abundantly in the cytoplasm of Schwann cells. Based on these observations, the biosynthesis of wildtype and Tr protein was examined in transfected cells. While wildtype PMP22 was readily transported to the plasma membrane, Tr protein was mainly localized in the endoplasmic reticulum (ER). Co-expression revealed a dominant effect of Tr on protein trafficking of wildtype PMP22. In agreement with the findings *in vitro*, Tr protein was not detectable in myelin of *Tr/0* mice.

6.1.2 Introduction

Mice carrying the autosomal dominant *Tr* mutation develop tremor, quadriparesis and transient seizures at a young age (Falconer, 1951). The mutation affects the peripheral nervous system (PNS) and manifests as a Schwann cell defect characterized by severe hypomyelination and continuos Schwann cell proliferation throughout life (Aguayo et al., 1977; Perkins et al., 1981). Supernumerary Schwann cells in the peripheral nerves of *Tr* form onion bulb structures around thinly myelinated axons, most likely as the result of repeated cycles of demyelination and remyelination (Ayers and Anderson, 1973). Heterozygous *Tr* mice retain approximately 5-30% myelinated fibers (Inuzuka et al., 1985), whereas homozygous animals are virtually devoid of PNS myelin and develop a more severe phenotype (Henry and Sidman, 1988). Molecular genetic analysis of *Tr* revealed a point mutation, co-segregating with the phenotype, which leads to a amino acid substitution from glycine to aspartic acid in a putative transmembrane domain of the PMP22 protein (Suter et al., 1992a). Similar mutations have been found to be associated with the hereditary human peripheral neuropathy Charcot-Marie-Tooth disease type 1A (CMT1A) (Valentijn et al., 1992b; Roa et al., 1993a; Roa et al., 1993c; Navon et al., 1996), the congenital hypomyelinating peripheral neuropathy Dejerine-Sottas syndrome (DSS) (Roa et al., 1993b; Ionasescu et al., 1995; Valentijn et al., 1995) and a second spontaneous mouse mutant, *Trembler-J* (Suter et al., 1992b). The most common form of CMT1A, affecting 70-80% of all CMT patients, is linked to a 1.5 megabase intrachromosomal duplication on chromosome 17p11.2 (for review, see Suter and Snipes, 1995). The affected chromosomal region contains the *PMP22* gene, suggesting that overexpression of PMP22 underlies CMT1A (Lupski et al., 1991; Raeymaekers et al., 1991; Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992a). This hypothesis has been confirmed through the analysis of transgenic rats and mice that carry additional copies of the PMP22 gene (Magyar et al., 1996; Sereda et al., 1996). Deletions of the same chromosomal segment that is duplicated in CMT1A are linked to the mild hereditary neuropathy with liability to pressure palsies (HNPP) (Chance et al., 1993). The causative role of PMP22 in HNPP

has been directly demonstrated by the generation of PMP22-deficient mice that exhibit a phenotype comparable to the human disease (Adlkofer et al., 1995). Furthermore, recent quantitative analysis of PMP22 expression by immunogold labeling techniques on biopsies of CMT1A and HNPP patients revealed increased and decreased PMP22 expression, respectively (Vallat et al., 1996).

PMP22 is a small glycoprotein expressed mainly by Schwann cells and localized in compact myelin (Snipes et al., 1992). It belongs to a family of integral membrane glycoproteins with four predicted transmembrane domains (Marvin et al., 1995; Taylor et al., 1995; Ruegg et al., 1996; Taylor and Suter, 1996) and is structurally related to a diverse group of potential four-helix bundle proteins localized in myelin (Snipes and Suter, 1995). Although PMP22 was identified as a peripheral myelin protein that is downregulated after injury-induced denervation, PMP22 expression is not restricted to myelinating Schwann cells (Kitamura et al., 1976; Spreyer et al., 1991; Welcher et al., 1991). PMP22 transcripts and protein have been detected in various adult tissues, most notably in the brain, intestine, lung, heart (Patel et al., 1992; Taylor et al., 1995) and motoneurons (Parmantier et al., 1995). Furthermore, PMP22 mRNA expression is widespread during mouse embryonic development (Baechner et al., 1995). Two alternative promoters control tissue-specific expression and regulation of PMP22. The distal promoter is specifically activated in myelinating Schwann cells, while the more proximal promoter was found to be active in all known PMP22-expressing tissues (Bosse et al., 1994; Suter et al., 1994). Although it became evident from the analysis of transgenic animals with altered PMP22 expression that correct dosage of PMP22 is crucial for the development and maintenance of peripheral nerves, the exact function of PMP22 remains elusive (Suter and Snipes, 1995). *In vitro* approaches have suggested that PMP22, in addition to its role as a component of compact PNS myelin (Snipes et al., 1992; Haney et al., 1996) as a potential adhesion molecule (Snipes et al., 1993), may have a more ubiquitous function in the regulation of the cell cycle and apoptosis (Manfioletti et al., 1990; Fabbretti et al., 1995; Zoidl et al., 1995).

The *Tr* mouse has already served as the key to the discovery of the genetic basis of hereditary motor and sensory neuropathies (Suter et al., 1992a; reviewed by Suter et al., 1993). Thus, we have chosen this model system to search also for potential disease mechanisms. In this report, we demonstrate that the *Tr* phenotype involves impaired intracellular trafficking of Tr protein and its dominant-negative effect on the transport of wildtype PMP22.

6.1.3 Results

PMP22 expression in *Tr* sciatic nerves

Although extensive morphological studies have been performed on peripheral nerves of *Tr* animals (Ayers and Anderson, 1973; Low and McLeod, 1975; Perkins et al., 1981; Koenig et al., 1991), the role of the mutated PMP22 protein underlying the disorder remained unclear (Suter et al., 1992a). Therefore, we have compared the expression of PMP22 in the sciatic nerve of eight month-old heterozygous *Tr/+* mice and wildtype control littermates by confocal microscopy (Figure 3). Ten µm consecutive, cross- and longitudinal cryosections were stained with polyclonal rabbit antibodies against PMP22 (Figure 3; A to D), the ER-marker OST48 (Figure 3; E to H) and P0 (Figure 3; J to L). In control animals, staining for PMP22 (Figure 3; A and C) and P0 (Figure 3; J and K) was indistinguishable and restricted to compact myelin while OST48 immunoreactivity appeared mainly concentrated in close proximity to the axons (Figure 3; E and G). As expected, a significant increase in Schwann cell nuclei (visualized by staining with propidium iodide) was observed in the *Tr/+* mutant (Figure 3; B, F, I and D, H, L) (Perkins et al., 1981). Myelinated axons in *Tr/+* mice were stained for PMP22 and P0 (Figure 3; arrowheads) but the myelin sheaths appeared too thin, in agreement with previous morphological analysis (Low, 1976). Surprisingly, PMP22 but not P0 was also detected abundantly in the cytoplasm of Schwann cells not associated with myelin sheaths (Figure 3; B and D, arrows), in a pattern reminiscent of the ER marker (Figure 3; F and H, arrows), suggesting a specific disturbance of PMP22 protein trafficking in *Tr/+* Schwann cells.

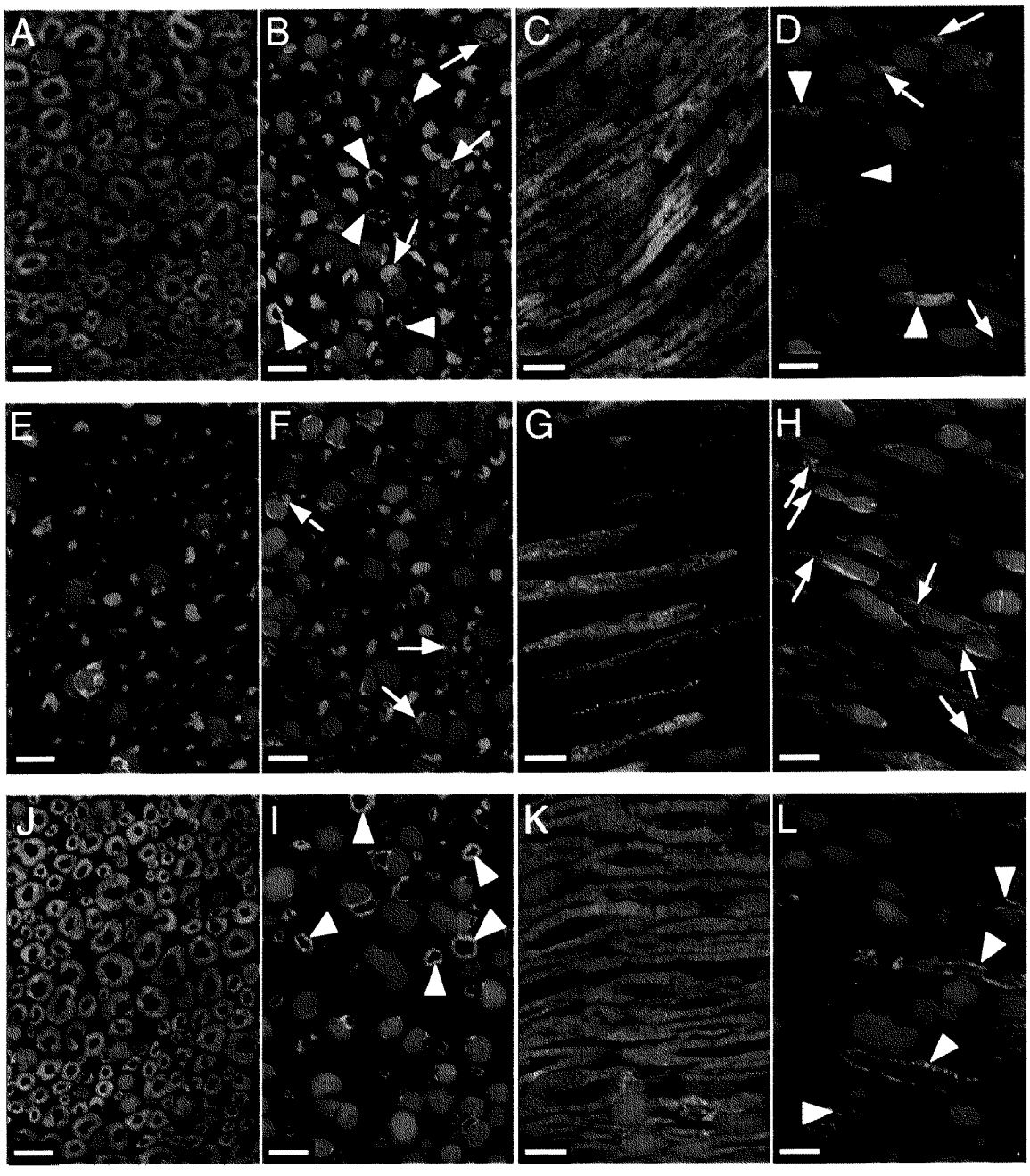

Figure 3: Localization of PMP22 and P0 in wildtype and *Tr/+* mice.

Confocal micrograph of the expression pattern of PMP22 (A to D; green), ER marker OST48 (E to H; green) and P0 (J to L; green) in cross- (two left panels) and longitudinal- sections (two right panels) of sciatic nerves from 8 month-old *Tr/+* (B, F, I and D, H ,L) and wildtype control littermates (A, E, J and C, G, K). Axons are stained for neurofilament H (blue), nucleic acids are stained with propidium iodide (red). In the wildtype, PMP22 (A and C) and P0 (J and K) immunoreactivity is restricted to compact myelin. In the *Tr/+* mutant, approximately 30% of the axons are thinly myelinated and show staining for PMP22 (B and D; arrowheads) and P0 (I and L; arrowheads). In contrast to P0, PMP22 is also detected in the cytoplasm in close proximity to the nucleus (B and D; arrows), in a pattern reminiscent of the ER marker OST48 (F and H; arrows). Bar represents 10 μm.

Transport and processing of PMP22 and Tr in transfected cells

To corroborate our initial *in vivo* observations in a suitable *in vitro* model system and to investigate the cellular mechanism underlying the effects of the *Tr* mutation, we transiently transfected COS-7 cells with mammalian expression constructs for the PMP22 and Tr proteins and analyzed intracellular trafficking.

Transfected cells were stained with a PMP22-specific antibody and protein distribution was visualized by immunofluorescence microscopy (Figure 4). Both proteins were readily produced and PMP22 was transported to the plasma membrane as confirmed by staining of plasma membrane microvilli (Figure 4; A, arrowhead). Tr protein staining, however, was restricted to intracellular compartments reminiscent of an expanded ER (Figure 4; C, insert). This effect was not cell-type specific since similar results were obtained with the human cervix carcinoma line HeLa and the rat glioma cell line C6 (data not shown).

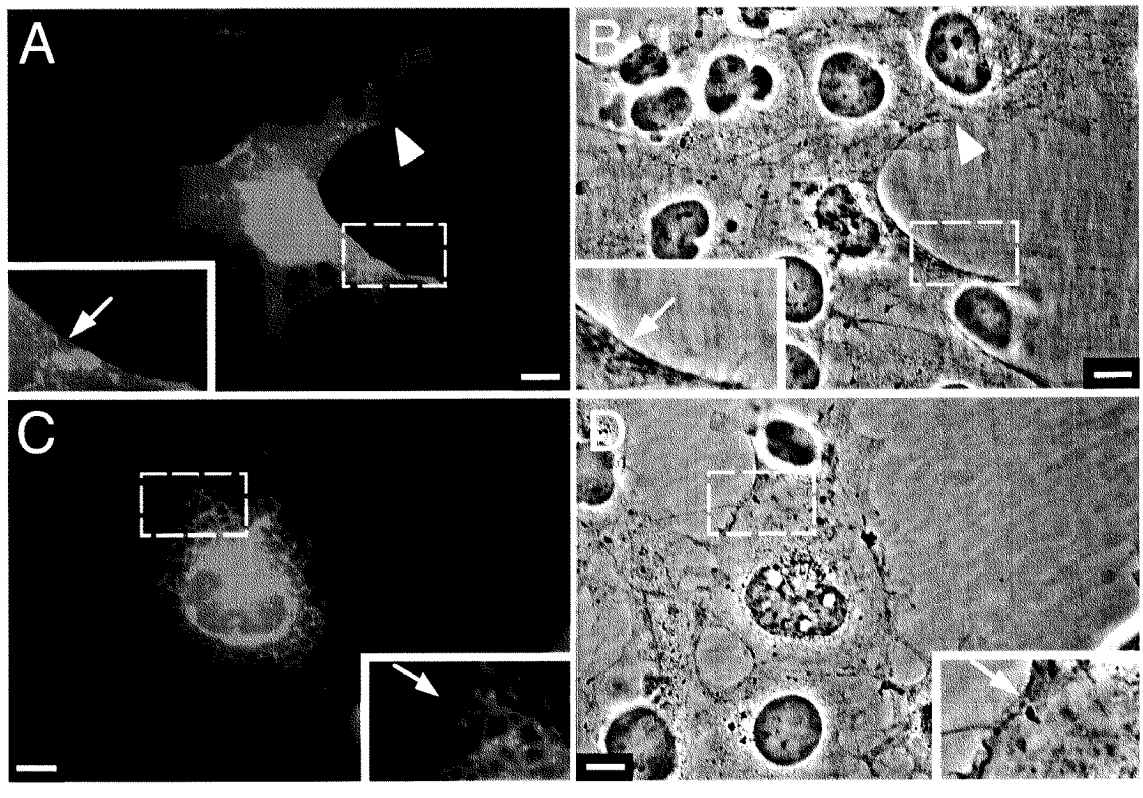

Figure 4: Expression of wildtype and Tr protein in transfected COS-7 cells.

Wildtype PMP22 protein is transported to the plasma membrane of the cell (arrows point to the plasma membrane border), particularly evident by staining of microvilli (A; arrowhead), while Tr

Double stainings with antibody markers specific for intracellular compartments were performed to further clarify the cellular localization of PMP22 and the Tr protein. To optimize conditions for double stainings, we constructed epitope-flagged PMP22 (PMP22VSV) and Tr (TrVSV) cDNAs encoding seven amino acids of the vesicular stomatitis virus glycoprotein (VSV G) added to the C-terminus of PMP22. Flagged and wildtype proteins were both produced at comparable levels and glycosylated as determined by immunoprecipitation analysis (data not shown). Immunofluorescence staining patterns observed with anti-flag antibodies or PMP22 antibodies were indistinguishable (data not shown).

Confocal optical sections (0.5 µm) were analyzed to examine co-stainings of recombinantly expressed PMP22VSV- and TrVSV proteins with antibodies against the VSV-flag and markers for the ER (ID3) and the lysosomes (H4B4/Lamp2). PMP22VSV was transported through the ER and the Golgi apparatus to the plasma membrane which was strongly stained (Figure 5; A and C, green), and followed the endocytic pathway to the lysosomes (Figure 5; C, yellow). Partial co-localization was also observed with the ER marker, most probably due to detection of nascent chains of immature PMP22 protein (Figure 5; A, yellow). In contrast, TrVSV protein (Figure 5; B and D, green) remained almost completely retained in the ER which often appeared swollen and co-localized with the ER marker (Figure 5; B, yellow). Minor amounts of protein escaping to the Golgi apparatus were detectable by co-localization with Golgi-specific antibody markers (data not shown). TrVSV protein did not reach the plasma membrane or the endocytic pathway and the lysosomes (Figure 5; D, lysosomes in red). However, depending on the confocal section chosen, TrVSV protein co-localized in some cells slightly with the lysosomal marker in structures close to the nucleus, potentially representing proteasomes of the protein degradation machinery (data not shown; Jensen et al., 1995; Ward et al., 1995).

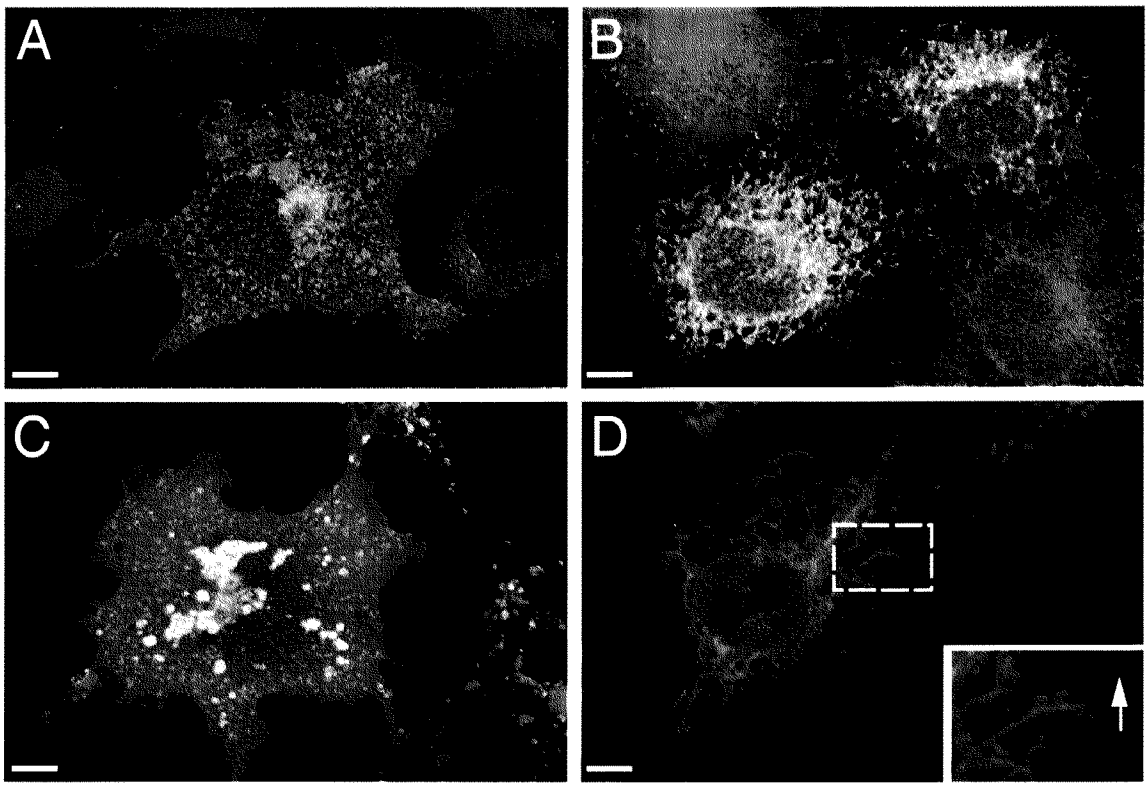

Figure 5: Subcellular localization of epitope-flagged wildtype PMP22 and Tr proteins in transfected COS-7 cells.

VSV-epitope flagged PMP22 (PMP22VSV: A and C; green) and Tr protein (TrVSV: B and D; green) were detected using VSV-epitope specific antiserum and co-staining for the ER marker ID3 (A and B; red) and the lysosomal marker H4B4 (Lamp-2) (C and D; red). Staining was visualized by confocal microscopy using 0,5 μm optical sections. PMP22VSV protein is transported beyond the ER to the plasma membrane (A and C; green) and follows the endocytic pathway to the endosomes/lysosomes where it co-localizes with Lamp-2 (C; yellow). In contrast, TrVSV protein remains intracellular and co-localizes with the ER marker ID3 (B; yellow). TrVSV protein does not reach the plasma membrane (B and D; green) or the lysosomes (D; red, arrow in insert). Untransfected cells are stained for markers only (A to D; red). Bar represents 10 μm.

Dominant negative effect of the Tr protein on PMP22 transport

Since *Tr/+* animals are affected by severe PNS dysmyelination, we hypothesized that the Tr protein might have a dominant-negative effect on wildtype PMP22 protein transport. To address this question, we co-expressed PMP22VSV and TrVSV using equimolar plasmid ratios and compared protein trafficking by immunofluorescence microscopy. Cells were stained at various time points between 12 and 72 hours post-transfection to exclude a potential effect of PMP22 and Tr expression on the cell cycle and apoptosis as described for NIH3T3 cells (Fabbretti et al., 1995). However, we did not detect definitive signs of apoptosis by nuclear morphology in DAPI-stained cultures,

transfected cells (data not shown). We have chosen to perform the detailed analysis at 36 hours since at earlier time points, the frequency of detectable PMP22-positive cells was significantly lower, while later, the increased number of dead cells that detached from the substrate made accurate scoring for plasma membrane staining difficult. Nevertheless, the relative amount of dead cells compared to total transfectants was identical for both the wildtype and mutant expression constructs (data not shown).

Approximately 500 spread-out cells were scored for intracellular or plasma-membrane staining with the flag antibody (Figure 6). Approximately 80% of the cells expressing PMP22VSV were stained on the plasma membrane (Figure 6; open bar) while less than 1% of the cells expressing TrVSV showed plasma-membrane staining (Figure 6; filled bar). If PMP22VSV- and TrVSV plasmids were co-transfected, PMP22 immunoreactivity associated with the plasma membrane was seen only on approximately 15% of positively stained cells (Figure 6; hatched bar), in agreement with a dominant-negative effect of the Tr protein on PMP22 wildtype protein trafficking and the expected co-transfection rate of approximately 90% (Reid et al., 1991). To exclude the possibility that these results might be hampered by an unspecific effect of an overloaded and non-functional ER, we co-transfected an expression plasmid encoding the α-receptor subunit (IL-2Rα) of the IL-2 receptor complex at equimolar ratio with the TrVSV plasmid and double-stained the transfectants. In support of a specific effect of TrVSV on the transport of wildtype PMP22, the unrelated IL-2Rα membrane protein was readily transported to the plasma membrane while the TrVSV protein was still retained in the ER of cells expressing both proteins (data not shown). However, the dominant-negative effect of Tr protein on wildtype PMP22 protein trafficking *in vivo* is only partially effective since some axons in the sciatic nerve of *Tr/+* mice are wrapped by a thin myelin sheath which contains PMP22 immunoreactivity (Figure 3). Thus, a few *Tr/+* Schwann cells appear to be capable to overcome the block of intracellular PMP22 transport, or are less sensitive to this impairment, allowing limited myelination even in the presence of the *Tr* allele.

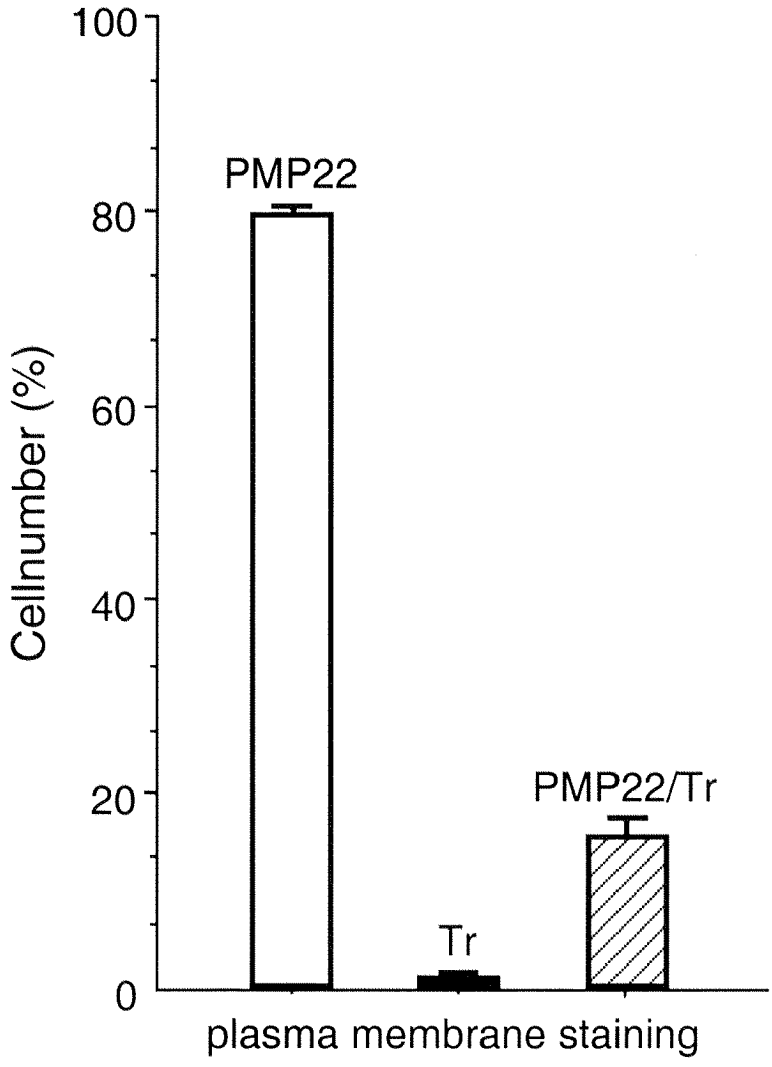

Figure 6: Effect of co-expression of TrVSV protein on PMP22VSV protein transport in transfected COS-7 cells.

Approximately 500 adherent cells transfected with equimolar amounts of PMP22VSV (PMP22), TrVSV (Tr) and the combination thereof (PMP22/Tr) were scored for intracellular and plasma membrane staining with an anti-VSV flag antibody (see Figure 5). Mean values and standard deviations of three independent transfections are shown.

Tr protein is not incorporated into compact myelin in *Tr/0* mice

To prove that Tr protein is indeed not transported to myelin, we have generated mice hemizygous for the *Tr* mutation by cross-breeding of *Tr/+* animals with heterozygous *pmp22* knock-out mice (Adlkofer et al., 1995; Adlkofer et al., 1997a). We analyzed PMP22 (Figure 7; A to C) and P0 (Figure 7; D to F) expression by confocal microscopy in consecutive cross sections of sciatic nerves of 18 day-old *Tr/0*, *Tr/+* and wildtype littermates. Sciatic nerves

of *Tr/0* animals contained only some myelin debris, as determined by electron microscopy (Adlkofer et al., 1997a). This residual myelin stained strongly positive for P0 (Figure 7; D) while consecutive sections were completely devoid of PMP22 immunoreactivity (Figure 7; A). In contrast to the striking ER-like PMP22 staining observed in eight months-old *Tr/+* animals (Figure 3; B), no obvious differences in PMP22 distribution relative to P0 were detected in 18 day-old mutants (Figure 7; B and E) mice.

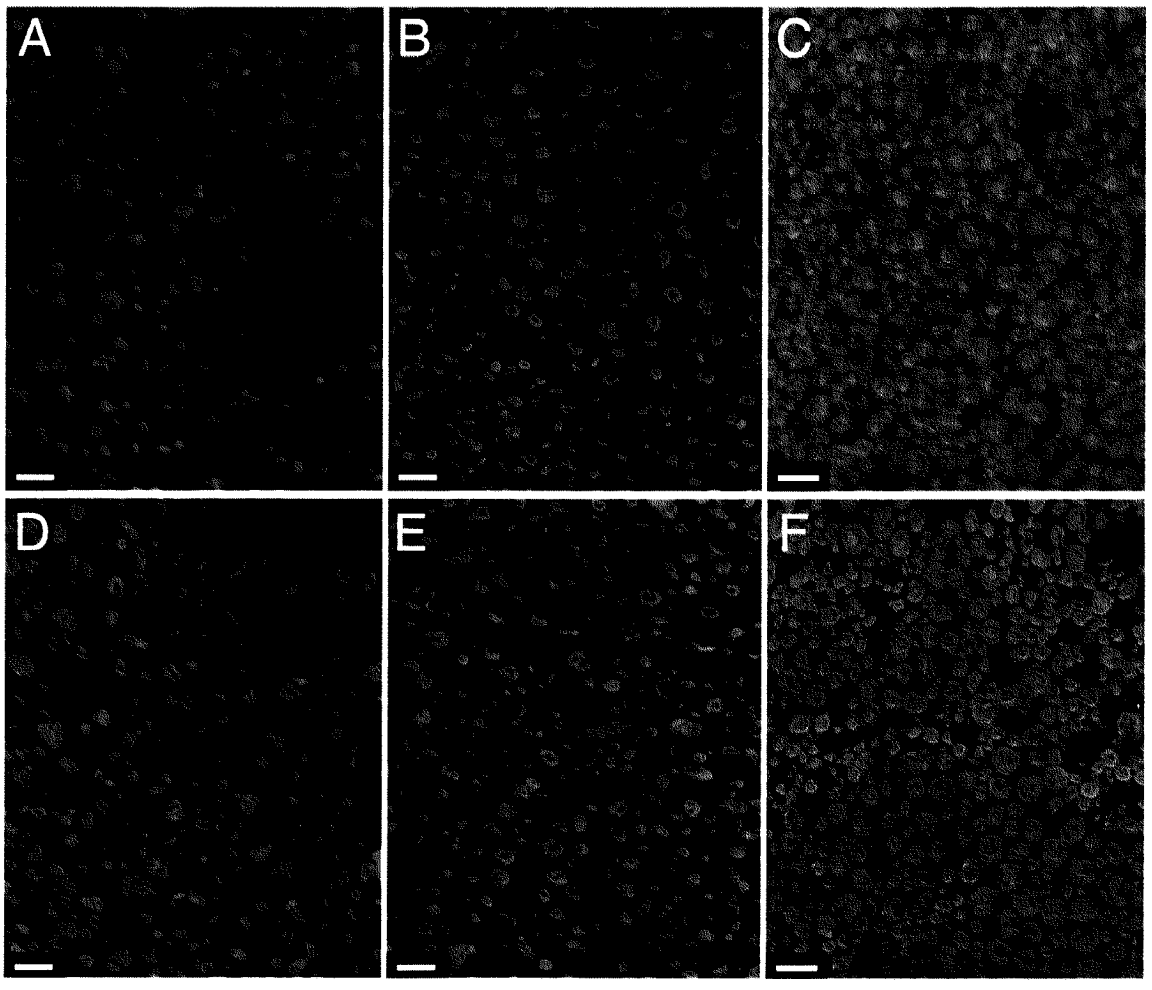

Figure 7: Lack of PMP22 protein in myelin of 18 day-old *Tr/0* mice.

Expression of PMP22 (A to C) and P0 (D to F) protein in cross-sections of sciatic nerves from 18-day old *Tr/0* (A and D), *Tr/+* (B and E) and wildtype (C and F) littermates (green). Axons are counter-stained for neurofilament H (red). Labeling was visualized by confocal microscopy using 0.5 μm optical sections. Both wildtype and *Tr/+* display no obvious differences in PMP22 localization (B and C) relative to P0 (E and F). In contrast, *Tr/0* nerves are completely devoid of PMP22 immunoreactivity (A), while residual myelin debris is strongly stained for P0 (D). Bar represents 10 μm.

6.1.4 Discussion

The *Tr* mouse represents a valuable animal model for a subgroup of the hereditary human peripheral neuropathies Charcot-Marie-Tooth disease type 1A and Dejerine-Sottas syndrome in which point mutations in the *PMP22* gene have been identified (reviewed by Suter and Snipes, 1995). In this report, we demonstrate impaired processing and transport of the Tr protein that is dominant over the correct intracellular trafficking of wildtype PMP22. Thus, we provide evidence for a potential cellular mechanism underlying some forms of neuropathies.

Our findings in transfected cells *in vitro* indicate that Tr protein cannot be transported to the plasma membrane and the complementary *in vivo* data demonstrating the lack of PMP22/Tr immunoreactivity in residual myelin in *Tr/0* mice support this hypothesis. However, we were unable to detect the mutant Tr protein in Schwann cells of 18 day-old *Tr/0* animals, possibly due to limited intracellular Tr protein accumulation at this age. This interpretation is supported by the observation that significant amounts of intracellular PMP22/Tr protein was only detectable in eight month-old but not in 18 day-old *Tr/+* mice. Unfortunately, a comparable analysis of older *Tr/0* mice was not possible since the animals died between the age of 18 and 24 days (data not shown). An alternative explanation for the failure to detect Tr protein in *Tr/0* mice suggests that the antibodies used in this study may not detect the Tr protein *in vivo*. This possibility seems unlikely since the recombinant Tr protein was readily detected in the ER of transfected cells. Finally, it might be argued that the *Tr* allele might not be expressed in *Tr/0* mice. This possibility is ruled out by the finding that the pathology observed in young *Tr/0* mice which are characterized by severe amyelination (data not shown) is fundamentally different from the focal hypermyelination (tomaculous neuropathy) phenotype observed in young $PMP22^{0/0}$ mice (Adlkofer et al., 1995). Thus, our data indicate that the *Tr* allele must be expressed by *Tr/0* mice in a fashion that is not compatible with normal myelination, possibly by causing some adverse effects to the physiology of the mutant Schwann cells.

Why is the Tr protein not transported to the plasma membrane? During biosynthesis, membrane proteins interact specifically with a set of chaperon proteins and folding enzymes, and an exquisite quality control mechanism restrains the transport of aberrant proteins through the biosynthetic intracellular compartments. If a protein is misfolded or abnormally oligomerized, it may not be incorporated in transport vesicles and targeted for degradation. Many mutations that lead to protein misfolding have been identified as the potential cause of human diseases (for review, see Thomas et al., 1995) by different cellular mechanisms, like loss-of-function through rapid degradation (e.g. cystic fibrosis (Cheng et al., 1990; Denning et al., 1992; Jensen et al., 1995; Ward et al., 1995)), toxic gain-of-function (e.g. Creutzfeld-Jakob disease (Brown et al., 1996) and Alzheimer's disease (Yankner et al., 1989; Harris et al., 1995; Suh et al., 1996)) or mislocalization of misfolded proteins or protein complexes (e.g. familial hypercholesterolemia (Hobbs et al., 1990) and Tay-Sachs disease (Lau and Neufeld, 1989)). In *Tr*, the available evidence shows convincingly that Tr protein causes peripheral neuropathy by a dominant-negative or gain-of-function mechanism, and the results presented in this report suggest a defect in intracellular transport to be involved in the disease mechanism. These combined findings reveal intriguing parallels to other myelin disease-associated proteins that share some structural similarities with PMP22. Mutations in the gene encoding the gap junction component connexin32 (Cx32) have been associated with the X-linked form of CMT (Bergoffen et al., 1993). In some families, female carriers are phenotypically affected suggesting a dominant disease mechanism, although X chromosome inactivation must also be considered (for review, see Suter and Snipes, 1995). It is anticipated, in analogy to our findings in *Tr*, that some of the mutant Cx32 proteins will be defective in intracellular transport and initial experimental support of this hypothesis has been reported (Omori et al., 1996). This finding is of particular relevance since the members of the connexin family form hexameric hemichannels that are assembled intracellularly and form functional connections with hemichannels on the plasma membranes of neighboring cells (for recent review, see Bruzzone et al., 1996). In Schwann cells, however, it has been suggested that gap junctions are formed between membrane lamellae of the same Schwann cell

in uncompacted regions of myelin, although hexameric subunits in a typical gap junctional array have yet to be demonstrated in the PNS (Scherer et al., 1995). Specific mutations in the Cx32 gene may interfere with the assembly of heteromeric and heterotypic channels potentially leading to a defect in intracellular transport. Alternatively, the interaction of mutated Cx32 with its potential partner proteins could alter the physiology of a Schwann cell, dramatically leading to a dominant-negative or gain-of-function effect. In analogy, one might speculate that PMP22 may also form multimeric complexes that could be affected by either the incorporation of misfolded mutant PMP22 protein, or increased or decreased availability of PMP22 protein due to altered gene dosage causing a deleterious effect on Schwann cells. Such a hypothesis is supported by transgenic mice carrying additional copies of the *pmp22* gene which show dramatic effects on myelination due to impaired Schwann cell differentiation (Magyar et al., 1996). A delay in myelination was also observed in other PMP22 mutant animals and P0-deficient mice suggesting that myelination initiation may require the formation of a heteromeric "promyelination" complex of several myelin proteins (Aguayo et al., 1977; Perkins et al., 1981; Giese et al., 1992; Adlkofer et al., 1995; Martini et al., 1995; Magyar et al., 1996; Sereda et al., 1996). Such a complex is likely to be assembled intracellularly and its formation would be more dramatically affected by the incorporation of a misfolded PMP22 protein than the lack thereof, providing an alternative explanation for both, the dominant effect of *Tr* compared to PMP22-deficient mice, and the clinically more severe phenotypes of CMT1A and DSS if compared to HNPP. Nevertheless, we were unable to detect accumulation of intracellular pools of P0 protein in both *Tr/+* and *Tr/0* animals, suggesting that the ER retention of mutated PMP22 protein could also lead to a more indirect alteration of Schwann cell physiology and subsequently influence their differentiation state as has been hypothesized for mutant PLP in oligodendrocytes (Jung et al., 1996).

The CNS myelin component PLP shares also some structural features with PMP22 (Suter et al., 1993). In striking similarity to the genetics of PMP22, mutations affecting PLP lead to classical and connatal Pelizaeus-Merzbacher disease (PMD) in human (Hodes et al., 1993; Griffiths et al., 1995;

Seitelberger, 1995) and similar CNS dysmyelinating diseases in other species (recent review, see Griffiths, 1996). In contrast, PLP-deficient mice show only a relatively minor defect in CNS myelin compaction (Boison and Stoffel, 1994; Klugmann et al., 1997). In agreement with these findings, evidence has been collected that mutated PLP leads to apoptotic cell death of oligodendrocytes, possibly through a toxic gain-of-function due to misfolding of the altered protein (Skoff, 1995). In agreement with these findings, Gow and colleagues have shown recently a direct correlation between disease severity of classical and connatal PMD and the transport ability of various PLP/DM20 mutant proteins *in vitro* (Gow et al., 1994a; Gow et al., 1994b; Gow and Lazzarini, 1996).

In conclusion, aberrant protein biosynthesis of potential tetraspan proteins is likely to emerge as a common feature in inherited myelination diseases including CMTX, PMD and a subgroup of CMT1A and DSS. It will be challenging to correlate the severity of clinical symptoms of CMT1A or DSS associated with different PMP22 mutations with the ability of the respective mutant proteins to be correctly transported. Furthermore, appropriate *in vitro* systems like the fast and efficient COS cell transfection paradigm described here may prove to be a useful addition to clinical diagnostics in order to distinguish disease-causing PMP22 mutations from DNA polymorphisms in small families and individual patients.

6.1.5 Experimental procedures

Animals

Heterozygous *Tr* mice were originally obtained from Dr. J.-M. Matthieu (University of Lausanne, Switzerland) and bred in our own conventional animal facility. Offspring was routinely genotyped by RT-PCR and *Eco*RV restriction digest as described (Suter et al., 1992a). *Tr/0* animals were generated by breeding of heterozygous *Tr/+* (CBA) and PMP22$^{+/0}$ (C57BL/6/AgoutiSv129EV) mice and double genotyped by PCR and genomic Southern blot analysis (Suter et al., 1992a; Adlkofer et al., 1995). Littermates were used as controls to minimize potential effects of the mixed genetic background.

Cell Cultures

COS-7 cells were cultured in DMEM (Gibco BRL) supplemented with 10 % FBS (Sera-Tech) and 50 µg/ml Gentamicin (Sigma) in a humidified atmosphere containing 6 % CO_2.

Expression Constructs

PMP22 and Tr cDNAs (Suter et al., 1992a) were subcloned into the *Hind*III and *Eco*RI sites of the mammalian expression vector pcDNA1 (InVitrogen), downstream of the cytomegalovirus promoter and designated pcDNA1PMP22 and pcDNA1Tr respectively. Epitope-tagged cDNAs were generated by PCR using the primers T7 up: 5'-AATACGACTCACTATAG-3' (corresponding to the T7 promoter upstream of the multiple cloning site of the pcDNA1 vector) and PMPVSV7 down: 5'-GCGGCCGCGGATCCTCTAGATCACTTTCCAAGTCGGT TCATCTCTGGTTCGCGTTTCCGCAGGATC-3' (corresponding to the nucleotides 462 to 480 of the open reading frame of the PMP22 cDNA followed by nucleotides encoding for the seven amino acids Glu-Met-Asn-Arg-Leu-Gly-Lys-Stop of the VSV G protein (Soldati and Perriard, 1991) and the endonuclease restriction sites *Bam*HI, *Xba*I and *Not*I). A proline residue was

inserted between PMP22 and VSV G protein sequences to ensure accessibility of the epitope tag. PCR reactions were performed with 1 ng of pcDNA1PMP22 or pcDNA1Tr as template and 500 ng of each primer using 1.25 units of AmpliTaq in PCR buffer II (Perkin Elmer). Samples were denatured for 2 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 55°C and 1 min at 72°C and a final elongation step at 72°C for 10 min. PCR products were separated on 1% agarose gels and purified with the GenClean II Kit (Bio 101) according to manufacturers instructions. Fragments were cloned *Hind*III and *Xba*I into the pcDNA1 vector and the entire DNA sequences were verified using the Sequenase 2.0 Kit (Amersham) according to manufacturer's instructions. Recombinant DNA was purified using Quiagen columns (Quiagen).

Transient Transfections

Exponentially growing COS-7 cells were trypsinized, washed in phosphate-buffered saline (PBS) and pelleted at 800 g. 1.5 x 10^6 cells were resuspended in 200 µl PBS containing 5 µg vector DNA in total, 2.5 µg each for the co-expression experiment. The cells were transferred to a 4-mm gap electroporation cuvette (Bio-Rad), chilled on ice for 5 min and electroporated at 300 V, 125 µF and 72 Ω (Electro Cell Manipulator 600 BTX, Electroporation System) resulting in pulse times from 6 to 10 msec. The transfected cells were chilled for 5 min on ice and split into seven 35-mm culture dishes containing 2 ml of medium and cultured for 12 to 72 h prior to analysis.

Tissue sections

Eight month-old *Tr/+* and 18 day-old *Tr/0*, *Tr/+* mice and corresponding wildtype littermates were sacrificed and sciatic nerves were removed, snap frozen in isopentane and embedded in OCT (Miles). 10 µm cross- and longitudinal sections were taken at -20°C (HM 500 Om microtom, Microm) and mounted on gelatin-treated slides.

Immunofluorescence

Tissue sections were thawed at 37°C and fixed immediately in acetone for 2 min at -20°C. Sections were washed 3 times in PBS containing 0.2% Tween 20 (PBS/T) (Sigma) for 10 min and permeabilized in methanol for 30 min at room temperature (RT) followed by 3 washes in PBS/T. Unspecific binding was blocked with 20% normal goat serum (Gibco BRL) in PBS/T for 1 hour at RT. Primary antibodies (see below) were diluted in blocking buffer and incubated overnight at 4°C, followed by 3 washes with PBS/T for 20 min at RT. Corresponding secondary antibodies (see below) were diluted in blocking buffer and incubated for 1 hour at RT. Specimens were washed 3 times in PBS/T for 20 min at RT and mounted in AF1 (Citifluor).

Cell cultures were rinsed twice with prewarmed DMEM buffered containing 10 µM HEPES and fixed with 2% paraformaldehyde for 30 min in the same buffer. Cells were washed 3 times for 20 min with Tris-buffered saline (TBS) at RT. Unspecific binding was blocked for 30 min at RT in TBS, 2% bovine serum albumin (Fraction V, Fluka), 0.1% porcine skin gelatin (type A, Sigma), 2% goat serum and the following detergents to ensure permeabilization: 1% Triton X-100 (Fluka) for PMP22-specific antibodies or 0.1% saponin (Sigma) for all other antibodies used (see below). Primary antibodies were incubated in blocking buffer overnight at 4°C, followed by 3 washes with TBS and incubation with secondary antibodies in blocking buffer for 1 hour at RT. Cells were washed 3 times in TBS for 20 min at RT and mounted in AF1(Citifluor).

Immunoreactivity was visualized by confocal microscopy using a Bio-Rad MRC-600 scanner in conjunction with a Zeiss Axiophot fluorescence microscope. Image stacks were obtained in single excitation mode to prevent spillover artifacts and recombined using Imaris and Selima image processing software (Bitplane AG, Technopark Zürich, Switzerland). Conventional fluorescence microscopy was documented using a Hamamatsu Colour Chilled 3CCD Camera in conjunction with Adobe's Photoshop 3.04 for the Macintosh.

Antibodies

Polyclonal anti PMP22 peptide 2 rabbit antiserum (Snipes et al., 1992; Pareek et al., 1993) was diluted 1:200 and stained plasma membranes of transfected cells only in the presence of 1% Triton X-100. Polyclonal anti-VSV G-protein rabbit antiserum Ra #49 11.5 (gift of Dr. J.-C. Perriard, Soldati and Perriard, 1991) was diluted 1:400, mouse monoclonal ER marker ID3 hybridoma supernatant (gift of Dr. S. Fuller) 1:10, mouse monoclonal lysosomal marker H4B4 hybridoma supernatant (Lamp 2, Developmental Studies Hybridoma Bank Iowa) 1:10 and worked best in the presence of 0.1% saponin. Polyclonal anti P0 rabbit antiserum Rabβ2 (gift of Dr. B. Trapp) was diluted 1:400, affinity purified ER marker OST48 polyclonal rabbit serum (gift of Dr. S. te Heesen) 1:50, rat monoclonal neurofilament marker NF-H hybridoma supernatant (gift of Dr. S. Scherer) 1:10 and were used in the presence of 0.2% Tween 20. Secondary antibodies donkey anti rabbit FITC, goat anti mouse TexasRed and goat anti rat TexasRed or Cy5 (Jackson) were diluted 1:100 in blocking buffer in the presence of the corresponding detergent.

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6.2 Impaired intracellular trafficking is a common disease mechanism of *PMP22* point mutations in peripheral neuropathies

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6.2.1 Abstract

The most common forms of hereditary motor and sensory neuropathies (HMSN) or Charcot-Marie-Tooth disease (CMT) are associated with mutations affecting myelin genes in the peripheral nervous system. A minor subgroup of CMT type 1A (CMT1A) is caused by point mutations in the gene encoding the peripheral myelin protein 22 (PMP22). To study the mechanisms by which these mutations cause the CMT pathology, we transiently transfected COS7 and Schwann cells with wildtype and PMP22 expression constructs carrying six representative dominant or *de novo* point mutations and one putative recessive point mutation. All but one of the first group of mutant PMP22 proteins failed to be incorporated into the plasma membrane and were retained in intracellular compartments of transfected cells. Surprisingly, the recessive PMP22 mutation produced a protein that was also mildly impaired in trafficking. Thus, our results suggest a common disease mechanism underlying the pathology of CMT1A due to PMP22 point mutations.

6.2.2 Introduction

Hereditary motor and sensory neuropathies (HMSN) are a large and heterogeneous group of disorders, that are classified in several subgroups based on clinical, electrophysiological and morphological criteria (for review, see Dyck *et al.*, 1993; Gabreels-Festen *et al.*, 1993). The most common form is Charcot-Marie-Tooth disease type 1 (CMT1) which is characterized by a dominant trait of inheritance and an estimated prevalence of approximately 1:10'000 (Nelis *et al.*, 1996). The onset of clinical symptoms of CMT1 patients is mainly in the second to third decade of life and is characterized by progressive weakness of the distal muscles with mild sensory impairment and markedly reduced nerve conduction velocities (NCV). Morphologically, nerves of CMT1 patients show demyelination, remyelination, onion bulb formation and loss of myelinated axons (Lupski *et al.*, 1991a). Recent advances in molecular biology have revealed several genetic defects leading to the CMT1 phenotype (reviewed in Snipes & Suter, 1995a). The most frequent form, termed CMT1A, is associated with a 1.5 megabase tandem duplication in the chromosomal region 17p11.2-p12 including the gene for peripheral myelin protein 22 (PMP22; Lupski *et al.*, 1991b; Matsunami *et al.*, 1992; Patel *et al.*, 1992; Raeymaekers *et al.*, 1991; Timmerman *et al.*, 1992; Valentijn *et al.*, 1992a). Based on these findings, it was proposed that a gene-dosage effect of PMP22 might be causative for the disease. This hypothesis has been confirmed by the generation of transgenic rodents carrying additional copies of the PMP22 gene and the quantitative analysis of biopsies of CMT1A patients (Huxley *et al.*, 1996,1998; Magyar *et al.*, 1996; Sereda *et al.*, 1996; Vallat *et al.*, 1996). Deletions of the same chromosomal segment that is duplicated in CMT1A are linked to the mild hereditary neuropathy with liability to pressure palsies (HNPP; Chance *et al.*, 1993; Lopes *et al.*, 1997). Formal proof for the crucial role of PMP22 dosage in this neuropathy has been gained from the analysis of genetically engineered PMP22-deficient mice (Adlkofer *et al.*, 1995,1997b). Furthermore, several distinct non-conservative point mutations of PMP22 (H12Q, L16P, M69K, S72L, S72W, S76I, S79C, S79P, L80P, G93R, G100R, L105R, G107V, T118M, L147R, G150D; for review, see De Jonghe *et al.*, 1997; Naef & Suter, 1998) have been identified in single

patients and families with different degrees of disease severity. According to the clinical classification, most of these patients have been diagnosed with severe CMT1A or Dejerinne-Sottas Syndrome (DSS or HMSN III). The latter disease entity was originally defined as a recessive peripheral neuropathy (Dejerine & Sottas, 1893; Gabreels-Festen *et al.*, 1993). However, DSS has lately been used more generally to describe a particular class of CMT1 with severe symptoms and early disease onset.

PMP22 is a 160 amino acid integral membrane glycoprotein and consists of four distinct hydrophobic domains and two extracellular domains (Figure 8; D'Urso & Muller, 1997a; Snipes et al., 1992; Snipes et al., 1993). It is predominantly expressed by myelinating Schwann cells but is also found in various neural and non-neural tissues (Baechner et al., 1995; Parmantier et al., 1995, 1997; Spreyer et al., 1991; Welcher et al., 1991). In the peripheral nervous system (PNS), PMP22 is confined mainly to the compact portion of myelin (Haney et al., 1996). Based on the CMT1A pathology and the morphological phenotypes of natural mutants and genetically engineered rodents, PMP22 appears to be involved in the initial phases of myelination, maintenance of myelin, and neuron-glia interactions (Adlkofer et al., 1995, 1997b; de Waegh et al., 1992). Furthermore, a crucial role of PMP22 in the regulation of cell proliferation and apoptosis has been suggested by cell culture experiments (D'Urso et al., 1997b; Fabbretti et al., 1995; Zoidl et al., 1995, 1997).

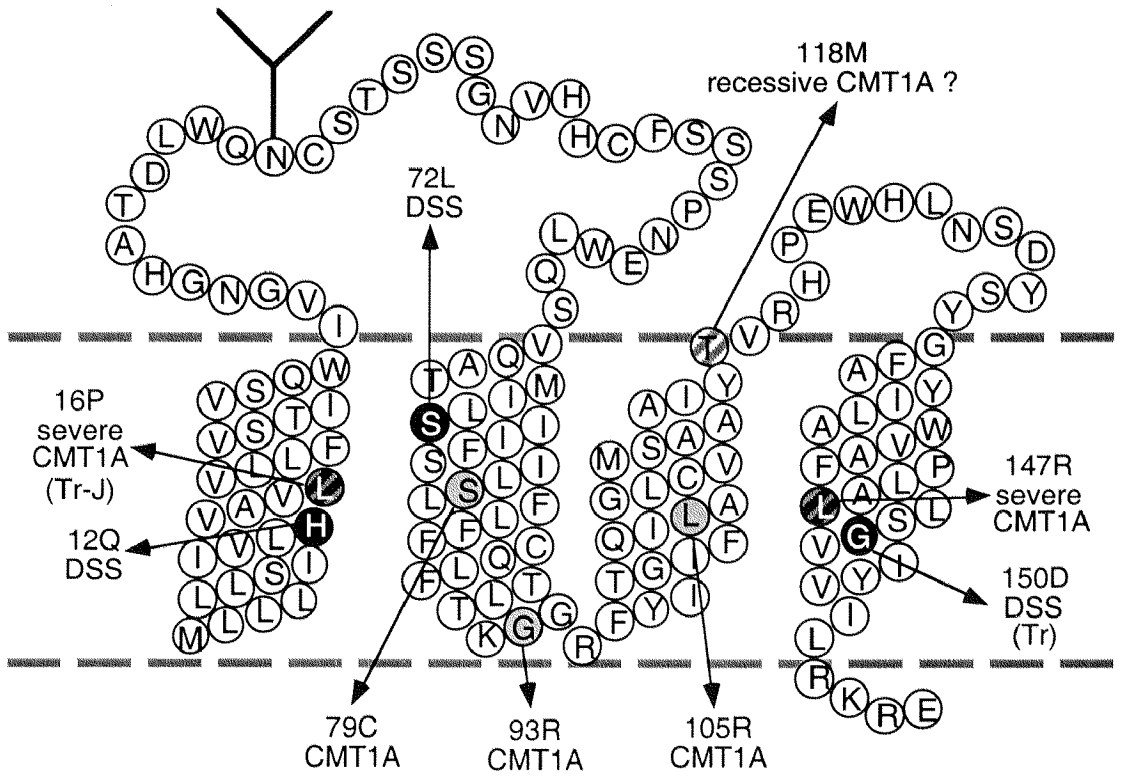

Figure 8: Representative disease mutations in a human PMP22 structure model.

Mutations from every structural unit of the molecule were chosen for analysis. Disease-causing mutations in the PMP22 protein are mainly found deep in the hydrophobic domains (for references, see text) with the exception of 93R that is flanked by basic amino acids and might protrude into the cytoplasmic space. Dark filled circles represent described Dejerinne-Sottas Syndrome (DSS) patients as used as synonym for the most severe phenotypes. Gray filled circles indicate Charcot-Marie-Tooth Disease type 1A (CMT1A) cases, and gray and black dashed circles represent intermediate phenotypes. A gray and white dashed circle marks the putative recessive mutation 118M. 72L and 79C represent mutations in the same hydrophobic domain but are described to cause different degrees of disease severity. 16P and 150D are shown since they were analyzed previously for protein trafficking (D'Urso *et al.*, 1998; Naef *et al.*, 1997; Notterpek *et al.*, 1997). Broad gray broken lines represent the borders of the plasma membrane with extracellular (e.g. intraperiod line in myelin) and the glycosylation site at position 41 (Y) on top and intracellular (e.g. major dense line in myelin) on the bottom.

PMP22 belongs to small family of highly related proteins including the epithelial membrane proteins (EMP)-1, -2 and -3 which share 33 to 43% amino acid identities and similar genomic organizations (Bolin *et al.*, 1997; Marvin *et al.*, 1995, Taylor *et al.*, 1995; Taylor & Suter, 1996, reviewed in Naef & Suter, 1998). Most notably, the hydrophobic, potentially membrane-associated regions (including most of the amino acid residues that are affected by PMP22 point mutations in CMT1A) are highly conserved. In addition, the consensus sequence for N-linked glycosylation is positioned

roles for this element. Furthermore, PMP22 has been proposed to represent the PNS counterpart of proteolipid protein (PLP), the main constituent of central nervous system (CNS) myelin, based on the similar putative membrane topologies and the involvement in myelination disorders with strikingly similar genetics (reviewed in Suter *et al.*, 1993).

The naturally occurring mouse mutants *Trembler* (*Tr*) and *Trembler-J* (*Tr-J*) suffer from a similar peripheral neuropathy as human CMT1A and DSS patients and led to the discovery of the involvement of PMP22 in these diseases (Suter *et al.*, 1992a, b). Both animals show non-conservative amino acid exchanges in hydrophobic domains of PMP22, G150D in *Tr*, L16P in *Tr-J*. Interestingly, the exactly identical mutations have been identified in human CMT1A and DSS patients (Ionasescu *et al.*, 1997; Valentijn *et al.*, 1992b). Recent studies have analyzed the mutations observed in *Tr* and *Tr-J* animals to identify the underlying disease mechanisms and revealed that improper protein trafficking is involved in this type of neuropathy (Adlkofer *et al.*, 1997a; D'Urso *et al.*, 1998; Naef *et al.*, 1997; Notterpek *et al.*, 1997). In the study presented here, we extended the search for disease mechanisms to additional PMP22 mutations that are associated with inherited peripheral neuropathies of different degrees of disease severity.

6.2.3 Materials and Methods

Cell Cultures

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10 % fetal bovine serum (FBS, Sera-Tech) and 50 µg/ml gentamicin (Sigma) in a humidified atmosphere containing 6 % CO_2.

Schwann cell cultures were prepared from neonatal rat sciatic nerve according to Brockes *et al.*, 1979, modified as previously described (Pareek *et al.*, 1993). Purified Schwann cells were grown on poly-L-lysine (Sigma) coated tissue culture dishes or glass coverslips in DMEM containing 10 % FBS, 50 µg/ml gentamicin, 100 µg/ml crude glial growth factor (GGF) and 5 µM forskoline (Sigma).

Mutant PMP22 expression constructs

All point mutations were inserted into pcDNA1mPMP22 (Naef *et al.*, 1997) using a PCR approach with primers containing the intended nucleic acid changes. The mouse cDNA was used as template for all mutations to allow reliable detection by the available PMP22 antibodies. H(12)Q was inserted in a one-step PCR using the primers H12Q-up 5'-GGGGATCCTGTTCCTGGGC ATCGCGG-3' containing the PMP22 internal *BamH*I endonuclease restriction site and SP6-down 5'-ATTTAGGTGACACTATA-3' (corresponding to the SP6 promoter downstream of the multiple cloning site of the pcDNA1 vector) and was cloned *BamH*I/*EcoR*I into the auxiliary vector pcDNA1mPMP22Bam containing the *Hind*III/*Bam*HI fragment from pcDNA1mPMP22.

All other mutated PMP22 cDNAs were constructed using a two-step overlap PCR strategy. Briefly, partial PMP22 cDNAs were amplified form pcDNA1mPMP22 in two independent first-step PCRs using the primers T7-up 5'-AATACGACTCACTATAG-3' (corresponding to the T7 promoter upstream of the multiple cloning site of the pcDNA1 vector) and S(72)L-down 5'-

GACTAACAGGATCATGGTGGC-3', A(79)C-down 5'-CAGACACAGGACGCTG AAG-3', G(93)R-down 5'-GCCGCGTTTGGTGAGAGTG-3', L(105)R-down 5'-AGCACGGATTTGGAAGAATCC-3', T(118)M-down 5'-CACCATGTAGATGGC CGCTGCAC-3' and L(147)R-down 5'-GA GGCGGGCTAGGGGAAAGG-3' to generate an upstream fragment. The corresponding downstream fragments were amplified using SP6-down 5'-ATTTAGGTGACACTATA-3' in conjunction with S(72)L-up 5'-CACCATGATCCTGTTAGTCATCTTC-3', A(79)C-up 5'-CTT CAGCGTCCTGTGTCTGTTCCTG-3', G(93)R-up 5'-CACTCTCACCAAA CGCGGCCGG-3', L(105)R-up 5'-GATTCTTCCAAATCCGTGCTGGTCTG-3', T(118)M-up 5'-GCGGCCATCTACATGGTGAGGCAC-3' and L(147)R-up 5'-CTTTCCCCTAGCCCGCCTCAGTG-3'. The resulting fragments were used as overlapping templates in the second-step PCR (Suter *et al.*, 1992c), amplified with the two outside primers T7-up and SP6-down and cloned *Hind*III/*EcoR*I into the pcDNA1 vector (InVitrogen). All VSV-epitope flagged PMP22 constructs were obtained using the primer PMPVSV7-down (Naef *et al.*, 1997) 5'-GCGGCCGCGGATCCTCTAGATCACTTTCCAAGTCGGTTCATCTCTGGTT CGCGTTTCCGCAGGATC-3' (corresponding to the nucleotides 462 to 480 of the open reading frame of the mPMP22 cDNA followed by nucleotides coding for the seven amino acids Glu-Met-Asn-Arg-Leu-Gly-Lys-Stop of the VSV G protein and the endonuclease restriction sites *Xba*I, *BamH*I and *Not*I) instead of SP6-down and fragments were cloned into the auxiliary vector pcDNA1mPMP22Bam.

First-step PCR reactions were performed with 1 ng of pcDNA1PMP22 as template and 500 ng of each primer using 1.25 units of AmpliTaq in PCR buffer II (Perkin Elmer). Samples were denatured for 2 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 55°C and 1 min at 72°C and a final elongation step at 72°C for 10 min. PCR products were separated on 1% - 2% agarose gels and purified using DE81 ion exchange paper (Whatman). Second-step PCRs were performed with purified first-step fragments as templates and the same conditions as the first PCR, except 2 min annealing time at 55° C. The entire sequences of the mutated cDNAs were verified using the Sequenase 2.0 Kit (Amersham) according to manufacturer's instructions.

Transient Transfections

Exponentially growing COS-7 cells were trypsinized, washed in phosphate-buffered saline (PBS) and pelleted at 800 g. 1.5 x 10^6 cells were resuspended in 200 µl PBS containing 5 µg vector DNA. The cells were transferred to a 4-mm gap electroporation cuvette (Bio-Rad), chilled on ice for 5 min and electroporated at 300 V, 125 µF and 72 Ω (Electro Cell Manipulator 600 BTX, Electroporation System) resulting in pulse times from 6 to 10 msec. The transfected cells were chilled for 5 min on ice and split into seven 35-mm culture dishes containing 2 ml of medium and cultured for 36 h prior to analysis. The average transfection frequency was 20 - 40 %.

Schwann cells were seeded on poly-L-lysine coated coverslips in a 24 well culture plate (Nunc) at 5 x 10^4 cells/well 24 hours prior to transfection. Cells were deprived of GGF and forskoline for two hours and transfected with 2 µg of DNA using polyethylenimine (PEI, Sigma) as a carrier. PEI was diluted to 100 mM with ddH2O and adjusted to pH 7.0 with 1 N HCl. Transfection mix was prepared for triplicate wells by premixing of 1.8 µl PEI and 6 µg of DNA in separate sterile tubes with 150 µl of a 150 mM NaCl solution each. DNA solution was added dropwise to the PEI solution, mixed carefully and incubated at room temperature (RT) for 5 min. Medium on the cells was replaced by 500 µl of Schwann cell medium and 100 µl/well of the transfection mix was added dropwise and distributed carefully over the entire well. Transfection efficiency was significantly increased by centrifugation of the plate for 5 min at 100 g at RT. Cells were incubated for 36 h prior to analysis. Average transfection frequencies were 5 - 15 %.

Immunofluorescence

Cell cultures were rinsed twice with prewarmed DMEM containing 10 µM HEPES and fixed with 2% paraformaldehyde for 30 min in the same buffer. Cells were washed 3 times for 20 min with Tris-buffered saline (TBS) at RT. Unspecific binding was blocked for 30 min at RT in TBS, 2% bovine serum albumin (Fraction V, Fluka), 0.1% porcine skin gelatin (type A, Sigma), 2%

goat serum and the following detergents to ensure permeabilization: 1% Triton X-100 (Fluka) for PMP22-specific antibodies or 0.1% saponin (Sigma) for all other antibodies used. Primary antibodies were incubated in blocking buffer overnight at 4°C, followed by 3 washes with TBS and incubation with secondary antibodies in blocking buffer for 1 hour at RT. Cells were washed 3 times in TBS for 20 min at RT and mounted in AF1 (Citifluor).

Immunoreactivity was visualized by confocal microscopy using a Leica TCS NT LCSM scanner in conjunction with a Leitz DM IRBE inverted fluorescence microscope. Image stacks were obtained in AOTF-optimized double excitation mode to prevent spillover artifacts and visualized using Imaris and Selima image processing software (Bitplane AG, Technopark Zürich, Switzerland). Conventional fluorescence microscopy was documented using a Hamamatsu Colour Chilled 3CCD Camera in conjunction with Adobe's Photoshop 4.01 for the Macintosh.

All mutants were expressed at similar levels as the wildtype protein and transfection rates were not significantly different (data not shown).

Antibodies

Polyclonal anti-PMP22 peptide 2 rabbit antiserum (Pareek *et al.*, 1993; Snipes *et al.*, 1992) was diluted 1:200. These antibodies stained plasma membranes of transfected cells only in the presence of 1% Triton X-100. Polyclonal anti-VSV G-protein rabbit antiserum Ra #49 11.5 (Naef *et al.*, 1997) was diluted 1:400, mouse monoclonal ER marker ID3 hybridoma supernatant (gift of Dr. S. Fuller) 1:10, mouse monoclonal lysosomal marker H4B4 hybridoma supernatant (Lamp 2, Developmental Studies Hybridoma Bank Iowa) 1:10 and worked best in the presence of 0.1% saponin. Secondary antibodies donkey anti-rabbit FITC and goat anti-mouse TexasRed (Jackson) were diluted 1:100 in blocking buffer in the presence of the corresponding detergent.

6.2.4 Results

Representative mutations

The analysis of the two PMP22 mutations L16P and G150D has previously been described in the context of the naturally occurring mouse mutants *Tr-J* and *Tr* (Suter *et al.*, 1992a, b), and aberrant protein trafficking is likely to be involved in the pathology of these peripheral neuropathies (D'Urso *et al.*, 1998; Naef *et al.*, 1997; Notterpek *et al.*, 1997). In humans, L16P was found in a non-duplication CMT1A family and was classified as severe CMT1A, based on the more severe neurological symptoms and reduced NCV compared to duplication CMT1A (Hoogendijk *et al.*, 1993). G150D was detected in two members of a family and was classified as DSS based on the severe clinical and electrophysiological symptoms and the early onset of the disease (Ionasescu *et al.*, 1997). To extend these studies, we have now evaluated whether alterations in protein trafficking are a common disease mechanism in peripheral neuropathies associated with PMP22 point mutations. We have chosen to analyze representative mutations from each motif of the predicted structure of the PMP22 molecule (Figure 8). Furthermore, we wanted to examine whether the precise location of a particular point mutation within PMP22 affects the disease mechanism, potentially explaining the observed differences in disease severity.

Analysis of PMP22 point mutations in protein trafficking

Expression and cellular sorting of mutated PMP22 proteins were analyzed in the non-neural COS7 cell line and cultured rat Schwann cells as a more natural host. PMP22 cDNAs containing the respective point mutations were constructed by PCR and were cloned into a mammalian expression vector. To optimize conditions for double stainings with antibody markers specific for intracellular compartments and to exclude crossreactivity with endogenously produced PMP22, a parallel set of constructs was also generated which encodes a seven amino acid epitope-flag at the C-terminus (Naef *et al.*, 1997). Transiently transfected cells were stained with specific antibodies for PMP22

and the epitope flag, respectively. Flagged and non-flagged proteins were both produced at comparable levels and the immunofluorescence staining patterns observed with anti-flag antibodies or PMP22 antibodies were indistinguishable in all mutants tested (data not shown). To investigate potential differences in protein trafficking between the different mutations, a statistical evaluation was performed.

In COS7 cells transfected with epitope-flagged wildtype PMP22 (PMP22VSV) 75 ± 3 % were stained on the plasma membrane (Figure 9, PMP22). In contrast, only 2 ± 1 % of the cells expressing the protein with the 12Q exchange showed detectable plasma membrane staining (Figure 9, 12Q). H12Q, located in the first hydrophobic domain of PMP22 (Figure 8), was identified as a *de novo* mutation in a single patient that had been diagnosed with DSS due to the severe motor impairment and drastically reduced NCV already at a very young age (Ouvrier *et al.*, 1987; Valentijn *et al.*, 1995).

The mutations S72L and S79C are both present in the second hydrophobic domain where most of the described PMP22 mutations are found (Figure 8, De Jonghe *et al.*, 1997; Naef & Suter, 1998). S72L was detected in three unrelated patients that were all classified as DSS based on severe neurological symptoms and virtually absent NCV (Ionasescu *et al.*, 1995, 1996; Roa *et al.*, 1993b). S79C was found in a single family with three affected members and was classified as CMT1A due to similar but somewhat lower NCV as found in CMT1A duplication patients (Roa *et al.*, 1993a). Cells transfected with 72L and 79C were positive for plasma membrane staining in 1 ± 0.5 % and 1 ± 0.3 % of the cells, respectively (Figure 9, 72L and 79C). Thus, both mutations lead to severe alterations in the transport of the corresponding mutant PMP22 proteins.

The clinical classification of the L105R mutation is not entirely clear. It was described in a single patient originally diagnosed with DSS based on the early onset of the disease and drastically reduced NCV (Figure 8, Gabreels-Festen *et al.*, 1994). However, this case was later redefined as CMT1A based on its genetics after the identification of the mutation in the third hydrophobic domain of PMP22 (Gabreels-Festen *et al.*, 1995). The examination of this mutant in our paradigm revealed plasma membrane staining of 1 ± 0.5 % of

the transfectants (Figure 9, 105L) indicating a strong impairment in protein trafficking.

L147R has been described as a de novo mutation in two members of a family and was classified as severe CMT1A based on clinical and electrophysiological features (Navon *et al.*, 1996). It is located in the fourth hydrophobic domain of PMP22 in close proximity to the *Tr* mutation (Figure 8). Again, this mutated protein was not transported to the plasma membrane of transfected COS7 cells since only 1 ± 0.1 % of the cells showed plasma membrane-associated staining (Figure 9, 147R).

The G93R alteration is the only mutation described so far to be associated with dominant CMT1A that is not located deep in a hydrophobic region of the protein (Figure 8). It is localized in the small loop between the large hydrophobic domains 2 and 3 and was classified as CMT1A based on morphological examination of a sural nerve biopsy (Ohnishi, 1995). Interestingly, G93R mutated protein behaved differently with respect to trafficking than the other PMP22 point mutations and statistical evaluation revealed only a slight reduction in cells positive for plasma membrane staining (Figure 9, compare PMP22 and 93R).

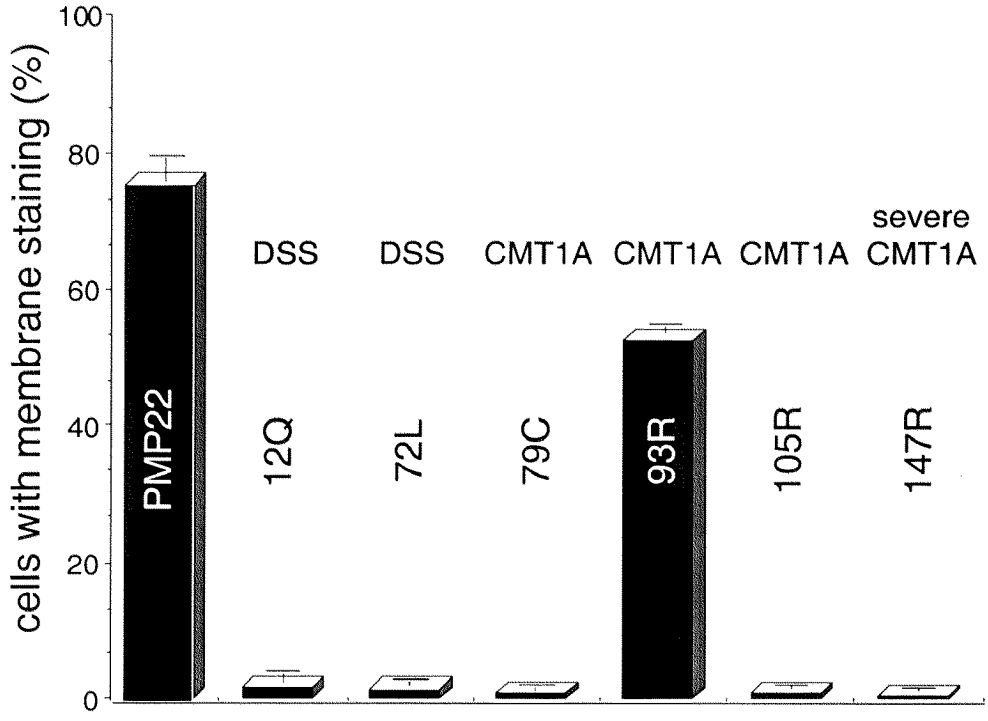

Figure 9: Impaired intracellular protein trafficking of mutated PMP22.

PMP22 mutations (12Q, 72L, 79C, 105R and 147R, for references, see text) located in all four hydrophobic domains (Figure 8) result in drastically impaired protein trafficking as demonstrated by the lack of plasma membrane staining of transfected COS7 cells compared to wildtype PMP22. In contrast, 93R is only slightly impaired with respect to protein trafficking. Cells transfected with the flagged expression constructs were examined 36 hours post transfection by immunofluoresence with the flag antibody and approximately 500 spread-out cells were scored for intracellular or plasma membrane staining. Mean values and standard deviations of three independent transfections are shown.

Subcellular localization of mutated PMP22 proteins

The subcellular localization of recombinant mutant PMP22 proteins was visualized by co-staining with the flag antibody and protein markers for the ER (ID3) and the lysosomes (H4B4/Lamp2) using confocal optical sections (0.3 µm) of transfected COS7 cells (Figure 10; COS). Staining was compared to conventional immunofluoresence images of transfected Schwann cells (Figure 10; SC) since the tested markers for distinct intracellular compartments failed to crossreact with their respective antigens in the rat cells.

PMP22VSV was transported through the ER and the Golgi apparatus to the plasma membrane which was strongly stained (Figure 10; A to C, green), and followed the endocytic pathway to the lysosomes (Figure 10; B, yellow). Partial co-localization was also observed with the ER marker, most probably due to detection of nascent chains of immature PMP22 protein (Figure 10; A, yellow). In contrast, 72L and 79C protein (Figure 10; D to F and G to I, green) remained almost completely retained in the ER which often appeared swollen and fragmented (Figure 10; D and G, yellow). 72L and 79C protein did not reach the plasma membrane (Figure 10; D to F and G to H) nor the endocytic pathway and the lysosomes (Figure 10; E and H, lysosomes in red). There was no obvious difference in subcellular sorting between DSS- (e.g. 72L, Figure 10; D to F) and CMT1A-causing (e.g. 79C; Figure 10; G to H) PMP22 variants. In contrast, the 93R mutation appeared virtually indistinguishable from wildtype protein with respect to protein trafficking (Figure 10; compare A to K, B to L and C to M).

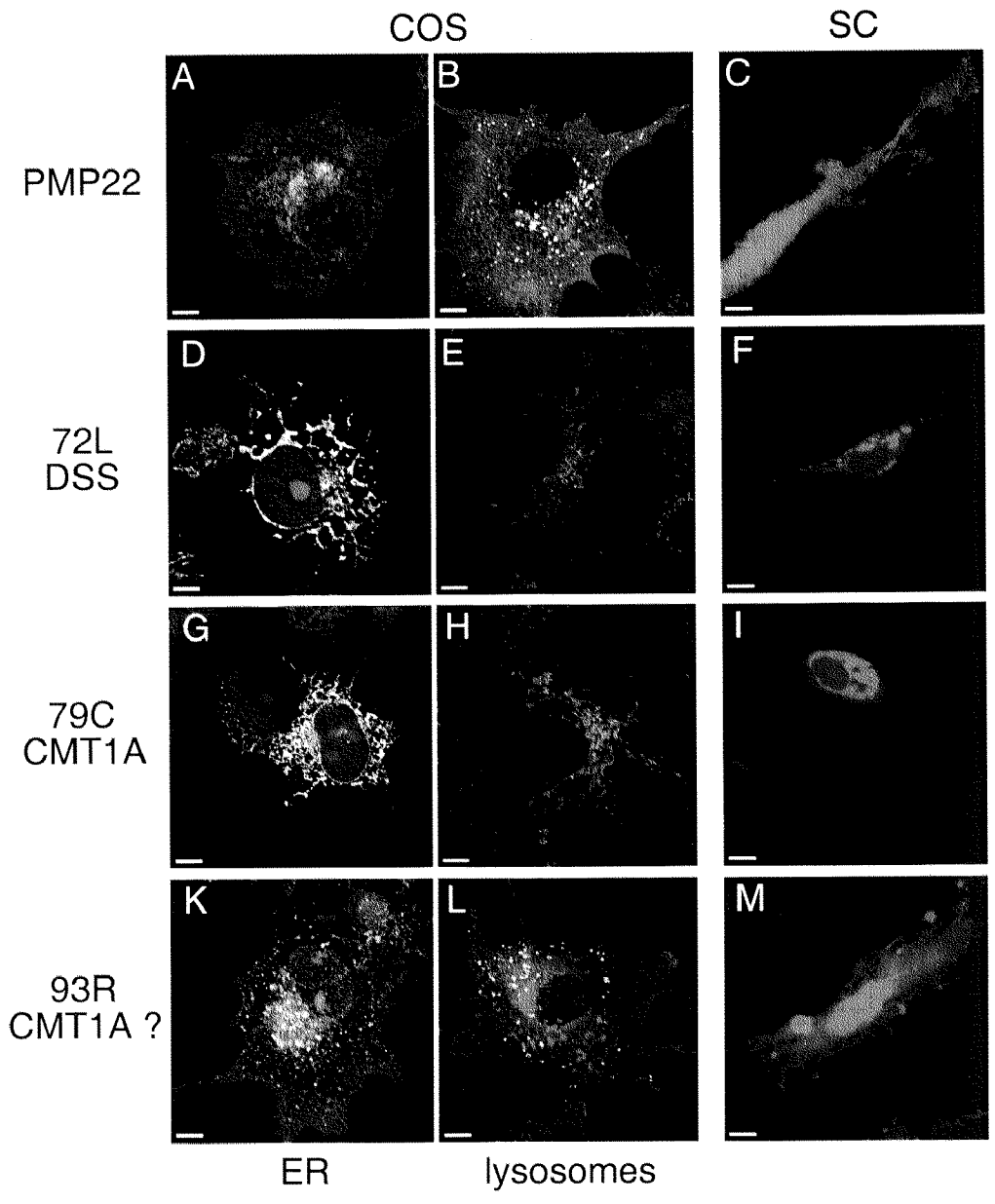

Figure 10: Subcellular localization of point mutated PMP22 proteins.

Localization of recombinant, epitope-flagged PMP22 proteins was visualized by co-staining with the flag antibody (FITC; appears green in all panels) and markers for either the ER (ID3; Texas red; appears red in 1. column) or the lysosomes (H4B4/Lamp2; Texas red in 2. column) using confocal optical sections (0.3 μm) of transfected COS7 cells (COS). Stainings are compared to conventional immunofluoresence images of transfected Schwann cells (SC). Wildtype PMP22 (A to C) and the mutants 72L (D to F) and 79C (G to I) as examples for DSS and CMT1A-causing mutations and the exception 93G (K to M) are shown. PMP22 is transported through the ER and the Golgi apparatus to the plasma membrane which is strongly stained (A to C, green), and follows the endocytic pathway to the lysosomes (B; appears yellow due to superimposition of the green and red signals). Partial co-localization with the ER marker is most probably due to detection of nascent chains of immature PMP22 protein (A, yellow). In contrast, 72L and 79C protein (D to F and G to I, green) remain almost completely retained in the ER which often appears swollen and co-localizes with the ER marker (D and G, yellow). 72L and 79C protein do not reach the plasma membrane (D to F and G to I) or the endocytic pathway and the lysosomes (E and H, lysosomes in red). There is no obvious difference in subcellular sorting between DSS- (72L, D to F) and CMT1A-causing (79C, G to I) mutations except for 93R. 93R behaves like the wildtype PMP22 protein with respect to protein trafficking (compare A to K, B to L and C to M).

T118M: A recessive mutation ?

The T118M mutation was found in a patient heterozygous for the HNPP deletion but classified as CMT1A based on the more severe clinical and electrophysiological symptoms compared to typical HNPP patients (Roa *et al.*, 1993c). This finding led to the hypothesis that T118M might be a recessive PMP22 point mutation that is only manifested in conjunction with the HNPP deletion. This idea has been challenged based on the discovery of a high allele frequency for the T118M mutation in northern Sweden and the lack of correlation to CMT1A (Nelis *et al.*, 1997). We have compared the T118M mutation with wildtype PMP22 in our transfection-based assay system. Surprisingly, while wildtype PMP22 protein gets transported to the plasma membrane (Figure 11, A), T(118)M remains mainly intracellular (Figure 11, B). This finding supports the interpretation that T118M behaves as a PMP22 missense mutation rather than an innocent polymorphism. However, statistical evaluation revealed a less pronounced effect for T118M on protein trafficking with 9 ± 1 % (Figure 11, C) of the cells showing plasma membrane staining compared to less than 2 % for all other PMP22 mutations examined (Figure 9).

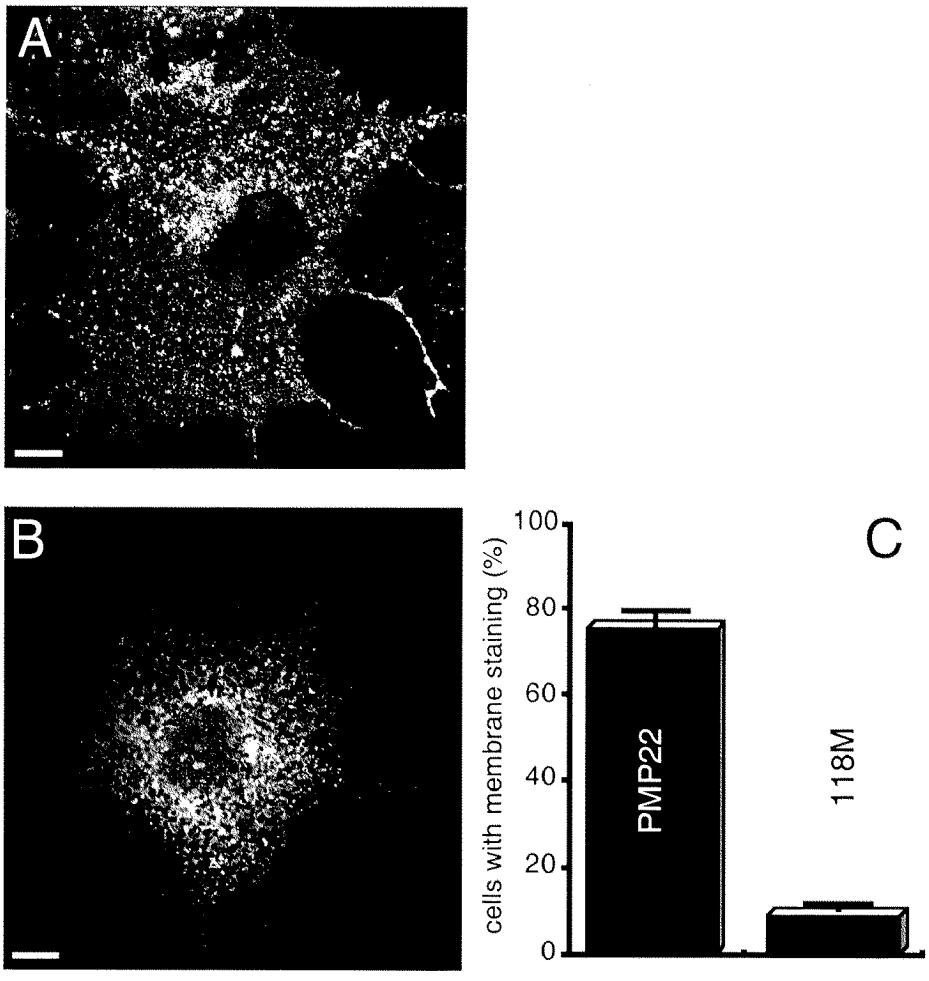

Figure 11: T(118)M: Evidence for a recessive PMP22 mutation.

Wildtype PMP22 protein is transported to the plasma membrane (A), while T(118)M remains mainly intracellular (B). 300 nm confocal optical sections of COS7 cells transfected with the flagged expression constructs and stained with the flag antibody are shown. Bar represents 10 μm. Statistical evaluation reveals some protein trafficking impairment effect for T118M in comparison to wildtype PMP22 (C, PMP22 and 118M) (compare to Figure 9).

6.2.5 Discussion

In an effort to understand the pathogenesis of PMP22 point mutations in CMT1A and DSS, we have compared protein trafficking of representative PMP22 mutations from the different structural units of the protein which result in different classes of clinical disease. We show severely impaired protein trafficking with apparent accumulation in intracellular compartments for all but one of the mutants in transfected COS7 and Schwann cells. Thus, we have identified a potential common disease mechanism for peripheral neuropathies caused by point mutations in PMP22. However, we were unable to decisively

correlate the clinical phenotype (as derived from descriptions in published reports) of the different mutations to their protein trafficking abilities except for the putative recessive T118M mutation. T118M behaved similar to missense mutations rather than a polymorphism although the effect was not as pronounced as for the other mutations. It should be noted in this context, however, that the clinical classification of PMP22 point mutations is often difficult since they are generally rare and often appear in small families. Furthermore, the definition of CMT1A and DSS needs refinement, since some investigators put the emphasis mainly on severity of symptoms and onset while others still use the original definition of a recessive, congenital hypomyelination. For example, the dominant or *de novo* PMP22 mutations analyzed in this work appear to be more severe than average duplication cases and should be classified as severe CMT1A based on the original definition.

G93R and T118M: Mutations or polymorphisms ?

The most surprising finding of this study is the different behavior of G93R with respect to protein trafficking relative to the other PMP22 point mutations tested. The question arises whether G93R is indeed a disease-causing mutation or just an innocent polymorphism. The 93R mutation has been identified only in a single patient (Ohnishi, 1995) supporting the latter hypothesis. On the other hand, G93R is the only described disease mutation located in a hydrophilic domain of the PMP22 protein and may therefore have less impact on protein folding and subsequent trafficking. It is conceivable that the small hydrophilic region between hydrophobic domain II and III defines a functional domain of the PMP22 protein. This hypothesis is supported by the fact that the glycine residue at position 93 is completely conserved in PMP22 species orthologues and the related EMP proteins, suggesting an important role of this specific amino acid in the structure and function of PMP22 and its relatives (Figure 12). Furthermore, a small reduction of the protein trafficking capacity was detected which may have a more significant impact *in vivo* than in our transfection paradigm (Figure 9). The final clarification of this controversial issue will require the identification of this mutation in a large CMT1A family or its reconstruction in transgenic rodents.

Our data further suggest that the T118M mutation is not a polymorphism and this notion is also supported through the conservation of this residue in PMP22 species orthologues and EMP-1 and EMP-2 (Figure 12). However, it remains unclear why T118M is only manifested in a compound heterozygous state in combination with a null allele (Roa *et al.*, 1993c). Since protein-trafficking deficiency was less pronounced compared to the other PMP22 mutations in our assay, the mutated protein may only be partially active. This interpretation would be consistent with the finding of a chronic CMT1-like phenotype in adult mice completely deficient of PMP22 (Adlkofer *et al.*, 1995).

Conservation of disease-related residues in PMP22 orthologues and family members

Careful analysis of the site and type of point mutations in PMP22 might help to resolve the open question of the molecular function of this protein. In fact, these mutations can be viewed as *in vivo* mutagenesis of PMP22 and therefore provide a tool to identify structurally and/or functionally crucial amino acids. Interestingly, almost all of the described disease-causing PMP22 point mutations are clustered in hydrophobic domains of the protein, indicating that those domains are vital for the correct function of PMP22 in the PNS (Figure 8). This interpretation is supported by the finding that most of the mutations affect amino acids that are conserved or conservatively exchanged in the species orthologues of PMP22 and even in the other members of the related EMP-family (Figure 12, black squares).

Strikingly, only very specific mutations in PMP22 appear to be disease-causing including the introduction of strong secondary structure-breaking residues, bulky amino acids and the introduction of charges in hydrophobic domains (Figure 12, last column). This interpretation is supported by the fact that two identical amino acid replacements (L16P, G150D) have been found in human and mouse even though the odds are slim for such an event to occur by chance (Ionasescu *et al.*, 1997; Suter *et al.*, 1992a, b; Valentijn *et al.*, 1992b). Based on these considerations, it is tempting to speculate that the mutated amino acids are crucial for the function of PMP22. On the other hand

it is likely that the alterations L16P, M69K, S79C/P, L80P, G100R, L105R L147R and G150D (Figure 12) cause also drastic changes in secondary protein structure that may result in misfolding and consequently hamper PMP22 function. It remains to be determined whether alternative changes at the same amino acid positions would be tolerated without effect or, in the other extreme (but less likely) case, are not compatible with a viable organism.

hPMP22	rPMP22	mPMP22	hEMP-1	hEMP-2	hEMP-3	Mutation, alteration
12[3] H	H	H	H	H	H	Q*, slightly more apolar
16[2] L	L	L	V	A	L	P#, helix break
69 M	M	M	M	M	M	K*, charge
72[3] S	S	S	S	S	S	L*/W*, apolar/bulky
76 S	S	S	C	C	C	I*, apolar
79[3] S	S	A	A	A	S	C#/P*, disulfid/helix break
80 L	L	L	L	F	F	P*, helix break
93[3] G	G	G	G	G	G	R#, charge
100 G	G	G	G	S	G	R*, charge
105[3] L	L	L	V	M	C	R#, charge
107 G	G	G	W	C	S	V#, conservative !
118[3] T	T	T	T	T	A	M+, slightly more apolar
147[3] L	L	L	F	F	L	R#, charge
150[1,2] G	G	G	G	G	G	D*, charge

[1,2,3]analyzed for protein trafficking
([1]Naef *et al.*, 1997 [2]D'Urso *et al.*, 1998, [3]this report)

* DSS, # CMT1A, + recessive CMT1A

Figure 12: Conservation of disease-associated residues in PMP22 orthologues and family members.

Relative positions of PMP22 residues affected in all described disease-causing mutations (for references, see text) and conservation of the amino acids in species orthologues and family members (h: human, r: rat, m: mouse). Complete conservation in all family members is indicated by black boxes. Exchanged amino acids and the corresponding potential biochemical alterations are shown in the last column.

Potential disease mechanisms

Point mutations can have many different adverse effects on protein function. Mutations in the active sites of enzymes can block the activity of the protein and lead to loss of function. Furthermore, the secondary and tertiary structure of proteins is determined by the primary amino acid sequence that can be disturbed by point mutations resulting in folding deficits. Proper folding is crucial for membrane proteins since during biosynthesis, membrane proteins interact specifically with a set of chaperon proteins and folding enzymes, and an exquisite quality control mechanism restrains the transport of aberrant

misfolded or abnormally oligomerized, it may not be incorporated in transport vesicles and is targeted for degradation. Many mutations that lead to protein misfolding have been identified as the potential cause of human diseases by different cellular mechanisms, like loss-of-function through rapid degradation, toxic gain-of-function or mislocalization of misfolded proteins or protein complexes (reviewed in Thomas *et al.*, 1995 and references therein).
Point mutations in PLP and PMP22 are associated with impaired protein transport (D'Urso *et al.*, 1998; Gow *et al.*, 1996, 1997b; Jung *et al.*, 1996; Naef *et al.*, 1997; Notterpek *et al.*, 1997; this study) and similar disease mechanisms have been reported for the gap junction protein connexin 32 (Cx32), the culprit gene for X-linked CMT (CMTX; reviewed in Scherer, 1997a, b). Cx32 is the third myelin disease-associated protein that shares some structural features with PMP22 and PLP and has been suggested to cause disease by similar mechanisms (Snipes & Suter, 1995). This hypothesis is supported by the demonstration of impaired protein transport of point mutated Cx32 proteins in transfected cells (Deschenes *et al.*, 1997; Yoshimura *et al.*, 1998) while Cx32-deficient mice develop mild structural abnormalities and reduced maintenance of peripheral nerve myelin with progressing age (Anzini *et al.*, 1997).

Loss-of-function due to PMP22 point mutations is unlikely based on the different clinical symptoms and pathology that are associated with the natural deletion found in HNPP and the comparative analysis of heterozygous PMP22 knockout mice and *Tr* animals (Adlkofer *et al.*, 1997a; Chance *et al.*, 1993). Furthermore, CMT1A caused by PMP22 duplication results in a similar disease phenotype compared to point mutations. These findings suggests that increased dosage and misfolding of PMP22 might lead to a disturbed equilibrium in the physiology of myelinating Schwann cells, possibly due to rate-limiting interactions with chaperones, or with other proteins that are transiently or steadily associated with PMP22. It has been demonstrated that only a small portion of the newly synthesized PMP22 protein acquires complex glycosylation and reaches the Golgi apparatus of cultured Schwann cells. The vast majority of PMP22 protein is retained in the ER and rapidly degraded. Surprisingly, this rapid intracellular turnover rate is not significantly altered in myelinating co-cultures of Schwann cells with sensory neurons or in

sciatic nerve explant cultures, although axonal contact promotes the translocation of PMP22 into myelin (Pareek *et al.*, 1993, 1997). It is tempting to speculate that misfolding is the reason for the abundant intracellular PMP22 degradation and such a mechanism would provide an explanation, why point mutations and increased gene-dosage may result in a similar cellular pathology in PLP/DM20 and PMP22 mutants by potentially increasing the amount of misfolded protein above a critical threshold level (Jung *et al.*, 1996; Suter & Snipes, 1995a). In the case of PLP, evidence has been collected that mutated PLP may lead to apoptotic cell death of oligodendrocytes, possibly through a toxic gain-of-function due to misfolding of the altered protein (Skoff, 1995). Alternatively, it cannot be excluded that PMP22 acts as a chaperone-like protein with an intracellular function itself, or that exact stochiometry and oligomerization is required for the correct intracellular transport and assembly of myelin components in general.

In summary, we have identified impaired protein trafficking as a potential common disease mechanism for CMT1A caused by point mutations in PMP22 in agreement with previous studies in *Tr* and *Tr-J* (D'Urso *et al.*, 1998; Naef *et al.*, 1997; Notterpek *et al.*, 1997). Six of seven mutant PMP22 proteins failed to be incorporated efficiently into the plasma membrane of transfected cells. It is anticipated that further systematic mutagenesis analysis will help to refine our current understanding of the structure and function of PMP22 and its family members in health and disease.

Acknowledgements

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7. Conclusion and Perspectives

In this work, we were able to identify improper protein folding and/or impaired processing as the major contributing factor to the disease mechanism of hereditary peripheral neuropathies due to point mutations in PMP22.

Starting from the naturally occurring *Tr* mouse carrying a G150D substitution in the PMP22 protein, we could demonstrate intracellular accumulation of PMP22-immunoreactive protein in Schwann cells of 8-month-old heterozygous animals. Recombinantly expressed Tr protein manly remains retained in the ER compartment of transfected COS-7 and cultured Schwann cells, most probably due to protein misfolding and/or impaired processing.

A similar behavior was demonstrated for the mutations H12Q, S72L, S79C, L105R, L147R that were identified in human patients suffering from severe forms of HMSN (severe CMT1A and DSS) and partially even for the alleged recessive T118M mutation using the same experimental paradigm. Only the putative CMT1A causing G93R substitution showed no intracellular retention, potentially because this mutation is not localized in a hydrophobic region of the PMP22 molecule. On the other hand this mutation was only identified in a single patient, therefore it cannot be excluded that this particular substitution represents a mere polymorphism.

Going one step further, we could also show that wildtype PMP22 protein processing and transport was impaired upon co-transfection with Tr protein in COS7 cells, potentially through hetero-dimerization and consequent interaction with the quality control mechanism of the ER/Golgi system (Tobler et al., 1999). The human mutations showed a similar dominant-negative effect on PMP22 protein trafficking with the exception of T118M and G93R (Figure 13). However, we cannot exclude that this effect is cell type specific for our experimental setup.

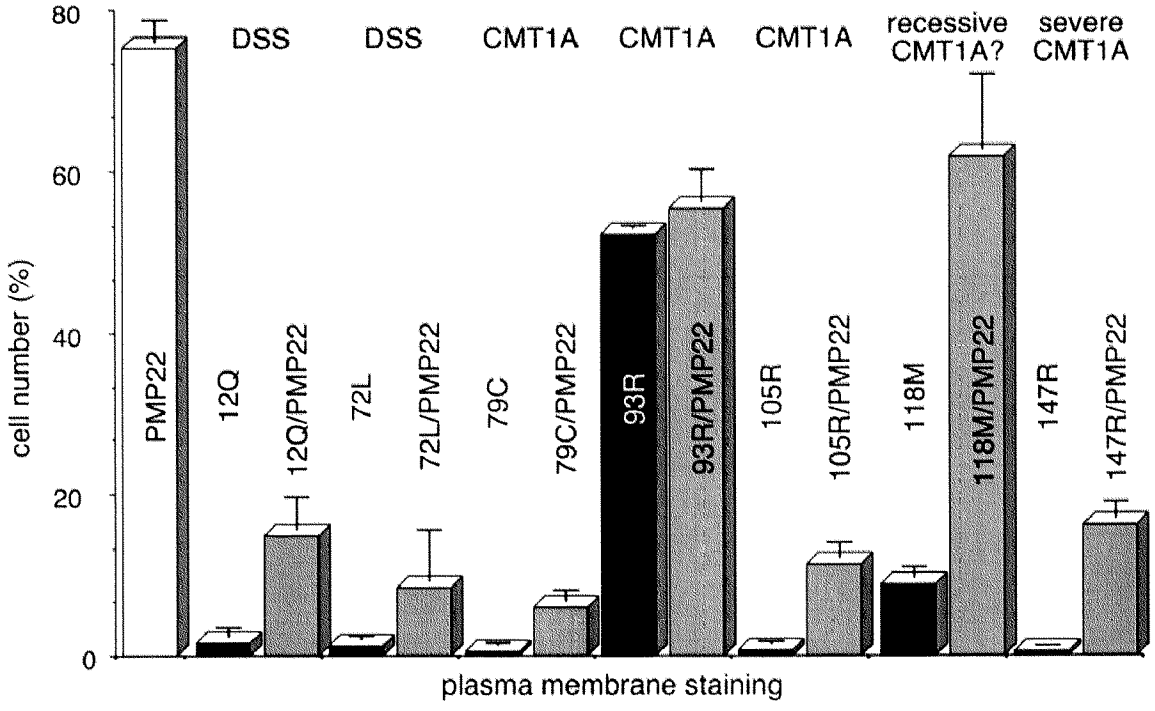

Figure 13: Effect of co-expression of point mutated PMP22 proteins on PMP22 transport in transfected COS-7 cells.

Co-expression of PMP22 mutations (12Q, 72L, 79C, 105R and 147R, for references, see text) drastically reduces protein trafficking of wildtype PMP22 protein in transfected COS7 cells as demonstrated by the lack of plasma membrane staining. 118M does not significantly influence PMP22 protein processing and behaves like a recessive mutation in this paradigm. In contrast, 93R is only slightly impaired with respect to protein trafficking and only slightly reduces PMP22 plasma membrane staining. The 10-20 % remaining cells positive for membrane staining in the co-transfections likely represent single PMP22 expressors as expected from 80-90 % efficiency in a co-transfection paradigm. Cells transfected with the flagged expression constructs were examined 36 hours post transfection by immunofluoresence with the flag antibody and approximately 500 spread-out cells were scored for intracellular or plasma membrane staining. Mean values and standard deviations of three independent transfections are shown.

In summary, we have identified improper protein folding and/or impaired processing as a common disease mechanism for hereditary peripheral neuropathies due to point mutations in the PMP22 protein.

Our conclusion is further supported by the analysis of the two other *pmp22* mutants *Tr-J* and *Tr-Ncnp*. Immuno-histochemical analysis on nerves derived from heterozygous *Tr-J* mice revealed an accumulation of PMP22 immunoreactivity in endosomal/lysosomal compartments of the mutant Schwann cells. Furthermore, the degradation of PMP22, P0 and MBP appears to be increased in these animals suggesting that the *Tr-J* mutation affects

myelin stability resulting in high myelin turnover and augmented degradation of myelin components by the lysosomal pathway (Notterpek et al., 1997). These data do not exclude, however, that the *Tr-J* protein is also affected by impaired protein trafficking (D'Urso et al., 1998; Tobler et al., 1999; Naef and Suter, unpublished data).

While *Tr* and *Tr-J* mice have been studied for decades, a third spontaneous autosomal dominant PMP22-mutant mouse strain named *Tr-Ncnp* has only recently been discovered (Suh et al., 1997). *Tr-Ncnp* is associated with an in-frame deletion of the entire exon IV of the PMP22 gene (Suter et al., 1994) resulting in an internal loss of 47 amino acids in the PMP22 polypeptide. Behaviorally, *Tr-Ncnp* mice show similar abnormalities to *Tr* and *Tr-J* including abnormal gait and ataxia. Myelination is initially normal but signs of segmental demyelination occur with progressing age (Suh et al., 1997). Like homozygous *Tr* mice (Henry and Sidman, 1988), homozygous *Tr-Ncnp* remain completely amyelinated but can survive for more than a year. However, the presence of giant vacuolar structures which are most prominent in Schwann cells of the sciatic nerve of homozygous animals are unique to *Tr-Ncnp* and are likely to represent abnormally swollen Schwann cell ER. Together with the finding of apoptotic cell nuclei in the affected nerves of *Tr-Ncnp*, these data have been interpreted that the *Tr-Ncnp* mutation may lead to the disruption of an intracellular transport system followed by Schwann cell death. Although the expression of *Tr-Ncnp* protein remains to be demonstrated in these animals, it is likely that the mutant protein is expressed since the phenotype of *Tr-Ncnp* mice is significantly different from mice carrying PMP22 null alleles. It emerges as a plausible hypothesis that similar mechanisms like the aberrant intracellular transport observed in *Tr* may also be functional in *Tr-Ncnp* mice (Naef et al., 1997). Whether there is additional impaired transport of other myelin components as a consequence of the PMP22 mutation in these mutant mice (as previously hypothesized for P0 and MAG in *Tr-J*; Heath et al., 1991) remains to be determined.

The generation and the analysis of animal models for *PMP22*-related neuropathies have confirmed many hypotheses, and they have greatly enhanced our current knowledge about the genetic causes and the patho-physiology involved. Nevertheless, many questions concerning the molecular functions and interactions of PMP22 remain open. In particular, recombinant expression approaches *in vitro* have suggested that PMP22 may be involved in the regulation of proliferation of Schwann cells and NIH3T3 fibroblasts (Muller et al., 1997; Zoidl et al., 1995; 1997). In addition, PMP22 might regulate apoptosis in NIH3T3 fibroblasts but not in Schwann cells (Fabbretti et al., 1995; Muller et al., 1997). Interestingly, the apoptosis-inducing effects were not observed when disease-causing mutations (including *Tr*) were assayed suggesting that this potential function of PMP22 may be dependent on the correct transport to the plasma membrane (Naef et al., 1997). Based on the *in vitro* data and the immuno-histochemical and morphological analysis of CMT1A biopsies, it was hypothesized that the defect in CMT1A may be due to a sequential series of events, starting with the genetic lesion of a *PMP22* gene duplication and followed by altered levels of PMP22 expression, progressing to the modulation of Schwann cell proliferation and abnormal differentiation, and culminating in defective myelination and altered myelin stability (Hanemann et al., 1996, 1998a, 1998b; Muller et al., 1997). The up-regulation of marker proteins for non-myelinating Schwann cells in PMP22-overexpressing mice lends some support to this idea (Magyar et al., 1996). On the other hand, cultured Schwann cells derived from transgenic rats with increased PMP22 gene dosage showed no obvious proliferation defects (Sereda et al., 1996), and a recent study using PMP22-overexpressing Schwann cells in an *in vitro* myelination system revealed no effect on initial myelin spiraling and myelin compaction (D'Urso et al., 1997b). It is important for the correct interpretation of the latter experiment, however, that the Schwann cells were isolated from newborn wildtype rats and have developed without altered PMP22 expression. This fact may explain the somewhat contrasting finding that marked myelin abnormalities were observed in myelinating organotypic cultures of *Tr* dorsal root ganglia (Mithen et al., 1982). The identification of early PMP22 expression in neural crest derived cell types and before myelination further supports the idea that PMP22 overexpression

may disturb Schwann cell development long before birth and the onset of myelination (Hagedorn et al., 1999; Notterpek et al., 1999).

It is anticipated that the careful quantitative analysis of Schwann cell proliferation, differentiation and apoptosis in the available PMP22 animal mutants will shed some light on the significance of these processes *in vivo*. In fact, initial analysis of biopsies from CMT1A and HNPP patients provide evidence for Schwann cell apoptosis in humans (Erdem et al., 1998). Similar findings were obtained from the analysis of PMP22 mutant mice (S. Sancho, unpublished observation) but it remains unclear how those events are linked molecularly to the observed distal axonopathy in these animals (Sancho et al., 1999). Additional transgenic experiments in rodents using the cre-recombinase system to obtain spatially and temporally controlled conditional overexpression or underexpression of PMP22 (Kühn et al., 1995) should allow the dissection of the potential differential effects of increased or decreased PMP22 expression on Schwann cell development, Schwann cell differentiation, myelination, and the maintenance of myelin and axons.

A key question for the understanding of the function of PMP22 is the strikingly similar phenotype of high PMP22 overexpression and mutations that alter the PMP22 protein. Although the detailed mechanisms have been shown to differ between the individual point mutations and overexpression (Notterpek et al., 1997; Tobler et al., 1999), some common fundamental basis is to be expected. We have demonstrated in the COS7 transfection system that all confirmed dominant PMP22 point mutations have a dominant negative effect on the transport of wildtype PMP22 protein in a similar fashion as previously shown for the Tr protein (Figure 13). Biochemical evidence for the formation of homo- and hetero-dimers and potentially also multimers of PMP22 and Tr-J protein (Tobler et al., 1999) further supports our hypothesis that the direct interaction of PMP22 with itself and other potential partner proteins is essential for the correct incorporation into the plasma membrane and subsequently into myelin. In this context, it will be important to examine the cause and consequences of intracellular retention of mutated PMP22 protein and whether PMP22 overexpression may also cause intracellular disturbances.

Additional questions that need to be addressed include: Is there a cellular response to the ER overloading and/or the potentially increased levels of misfolded or aberrantly oligomerized protein? Are chaperones or other PMP22-binding proteins titrated out (Neri et al., 1997)? Is such a process affecting the correct stochiometry of myelin components and/or their biosynthesis and translocation? These questions are of particular importance since there is growing experimental evidence for such interactions between PMP22 and P0 to occur (D'Urso et al., 1999). As a consequence, is the Schwann cell physiology continuously disturbed and intoxicated not allowing the maintenance of proper myelin? Is the potential function of PMP22 as a regulator of cell proliferation and cell death related to these processes? Other questions of more general importance include: How similar or dissimilar are Schwann cells and the myelin structure affected by altered gene dosage or mutations of its other components including P0 and connexin32 which are both mutated in other subtypes of hereditary neuropathies (Martini, 1997, Snipes and Suter, 1995)? Finally disease-oriented questions include: How is PMP22 involved in the etiology of other disorders like neuromyotonia (Toyka et al., 1997) or as a neuritogen in experimental allergic neuritis (Gabriel et al., 1998; Koehler et al., 1996; Weiss et al., 1999)?

Another important point of focus should be considered for additional experiments, since most disease related questions are addressed in complex mammalian *in vivo* systems. It may prove helpful to analyze "wildtype" PMP22 properties and interactions in more simplified and controllable experimental paradigms. The identification of PMP22/EMP-family homologues in the nematode C. elegans (Agostoni et al., 1999) and zebrafish (Wulf et al., 1999) provides promising systems for the research on the general functions of the PMP22/EMP-family of proteins independent of its functions in the myelin of the peripheral nervous system.

Using a cell-based *in vitro* approach, we have gathered initial evidence for the involvement of PMP22 in cell-cell adhesion and/or communication with the help of CHO cell lines that constitutively express PMP22 on the plasma membrane (unpublished observation). However, unstable expression levels prevented exact analysis of our clones, therefore it might be helpful to obtain

cell lines that express PMP22 in a controllable manner. On the other hand, PMP22 has withstood every approach to directly identify any of its proposed functions so far; therefore it may prove helpful to search for potentially interacting molecules to link PMP22 to already known biological pathways. An alternative cell culture based approach shows the first signs of success with the identification of a small GTPase RhoA-dependent regulation of cell spreading by PMP22 overexpression (Brancolini et al., 1999). Furthermore, it is conceivable that different structural elements of the PMP22 protein are responsible for the different proposed biological functions. To explore such functions it may be necessary to identify components of known biological pathways that directly interact with PMP22 or parts of it in order to link PMP22 to processes such as cell cycle regulation, apoptosis, cell adhesion or cell-cell communication. The yeast-two-hybrid system appears to be a suitable candidate for such an approach.

Additionally, recent progress in the biochemical purification of PMP22 protein (Sedzik et al., 1998) may lead to high resolution structural information that may consequently help to identify functions and/or functional domains of the protein. Furthermore, the biochemical and biophysical characterization and direct experiments with suspected interacting molecules will also become possible due to the availability of larger amounts of purified PMP22 protein.

PMP22 and its family members have only been studied intensively for approximately seven years and progress has been considerable. The necessary tools are now available to address the poignant questions, and we hope to learn a lot more about the structure and function of this fascinating group of proteins in health and disease in the years to come.

8. References

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10. Curriculum Vitae

Personal Data

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Date of Birth:	22.03.1966 from Hemberg SG, Switzerland
Nationality:	Swiss
Marital status:	single
Languages:	native German, fluent in English, good knowledge in French

Academic Education

1981-1985	*Matura*, Kantonsschule St.Gallen, Switzerland
1986-1992	*Dipl. sc. nat.*, Biochemistry, Molecular Biology & Biophysics, subsidiary subjects in Immunology, Biotechnology and Economics, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
1993-1999	Studying towards *Dr. sc. nat.*, Cell Biology, Neurobiology and Molecular Biology, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
1994-1995	Ph.D. student's delegate to the Board of Directors of the Institute of Cell Biology at the Swiss Federal Institute of Technology in Zürich

Research Positions

1998-present	Research and teaching assistant with Prof. Dr. T. Koller, Institute for Hygiene und Applied Physiology, Environmental Hygiene, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland

1993-1997	Graduate student and teaching assistant with Prof. Dr. U. Suter, Institute of Cell Biology, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
1992	Diploma student with Dr. H. U. Lutz, Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland

Professional Affiliations

1995-present	Member of the Swiss Society for Cell Biology, Molecular Biology and Genetics (USGEB/USSBE)

Publications

Giger, U., Sticher, B., **Naef, R.**, Burger, R. & Lutz, H. U. (1995). Naturally occurring human anti-band 3 autoantibodies accelerate clearance of erythrocytes in guinea pigs. Blood 85, 1920-8.

Naef, R., Adlkofer, K., Lescher, B. & Suter, U. (1997). Aberrant protein trafficking in Trembler suggests a disease mechanism for hereditary human peripheral neuropathies. Mol Cell Neurosci 9, 13-25.

Adlkofer, K., **Naef, R.** & Suter, U. (1997). Analysis of compound heterozygous mice reveals that the Trembler mutation can behave as a gain-of-function allele. J Neurosci Res 49, 671-80.

Pareek, S., Notterpek, L., Snipes, G. J., **Naef, R.**, Sossin, W., Laliberte, J., Iacampo, S., Suter, U., Shooter, E. M. & Murphy, R. A. (1997). Neurons promote the translocation of peripheral myelin protein 22 into myelin. J Neurosci 17, 7754-62.

Naef, R. & Suter, U. (1998). Many facets of the peripheral myelin protein PMP22 in myelination and disease. Microsc Res Tech 41, 359-71.

Cathomen, T., Mrkic, B., Spehner, D., Drillien, R., **Naef, R.**, Pavlovic, J., Aguzzi, A., Billeter, M. A. & Cattaneo, R. (1998). A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. Embo J 17, 3899-908.

Naef, R. & Suter, U. (1999). Impaired intracellular trafficking is a common disease mechanism of PMP22 point mutations in peripheral neuropathies.

Tobler, A. R., Notterpek, L., **Naef, R.**, Taylor, V., Suter, U. & Shooter, E. M. (1999). Transport of Trembler-J mutant peripheral myelin protein 22 is blocked in the intermediate compartment and affects the transport of the wild-type protein by direct interaction. J Neurosci 19, 2027-36.