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# FUNCTIONAL CHARACTERISTICS OF MYELIN PO PROTEIN MUTATIONS ASSOCIATED WITH CHARCOT-MARIE-TOOTH DISEASE

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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# ABSTRACT

## Functional Characteristics of Myelin Po Protein Mutations Associated with Charcot-Marie-Tooth Disease

by

Wenhui Li

Advisor: Professor Marie T. Filbin

Po, the most abundant protein of peripheral nervous system (PNS) myelin, is responsible for compaction of PNS myelin. The functional significance of this molecule is also manifest in patients suffering from the demyelinating neuropathy Charcot-Marie-Tooth (CMT) 1B (and its variants), as mutations in the Po gene are reported to be associated with this disease. However, despite the rapid progress in identification of those mutations in recent years, little is known about the disease mechanism. Since most CMT1B patients are heterozygous for mutations in Po, the aberrant Po may not reach myelin. Then the disease would be caused by insufficient Po. Alternatively, the mutated Po protein may reach myelin but could have a dominant-negative effect on the wild type Po.

Using an in vitro transfection/adhesion assay, we have shown that Po behaves like a homophilic adhesion molecule. This assay also enables us to test how individual mutations affect the functioning of Po. Three mis-sense mutations (Ser34 mutated to Cys (S34C), Asp61 mutated to Glu (D61E) and Lys67 mutated to Glu (K67E)), one deletion (Ser34 (ΔS34)) and two non-sense mutations (Tyr125 to stop codon (Y125X) and Tyr152 to stop codon (Y152X)), which each corresponds to a mutation found in patients, were

created in Po cDNA and then transfected into CHO cells. We showed that, except for Y125X and Y152X for which no Po protein was detected, all other mutated Po proteins reached the cell surface but failed to adhere. This suggests that these mutations abolish the adhesive properties of Po. From the three dimensional model of the extracellular domain of Po based on the crystal structure, it is possible that mutations at Asp61 and Lys67 affect the binding site directly while deletion of S34 could disrupt the protein conformation. Mutation of Ser34 to Cys could involve both possibilities.

As for the two non-sense mutations (Y125X and Y152X), as no protein was expressed, insufficient Po in myelin must be the cause of the disease. For the other four mutations, their ability to cause a dominant-negative effect on wild type Po was tested by co-expression of wild type and mutated Po in the same cell. The adhesive properties of these co-expressors were compared to those of cells expressing only the wild type Po. It was found that these mutated Po proteins all exerted a dominant-negative effect on the wild type of Po, however the severity of the effect varied from mild to blocking adhesion of wild type Po completely. These results suggest that the adhesion of wild type Po protein is partially or completely abolished by the presence of those mutated Po proteins. Furthermore, there is a correlation between the mutation and the disease phenotype. That is to say, mutation of Ser34 to Cys is shown to have the strongest dominant-negative effect of all the mutations tested, which correlates with its more severe disease phenotype. In contrast mild CMT1B phenotypes are associated with all the other mutations studies. This indicates that this assay system can be used as an indicator of the disease phenotype predicted from different Po mutations.

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# Introduction

# 1. Myelin

## 1.1. Myelin structure and function

The myelin sheath is one of the most remarkable features of the vertebrate nervous system. Large axons of neurons are wrapped by myelin which is a stack of highly specialized plasma membranes of particular glial cells. Myelinogenesis starts with spiral deposition of vast amounts of plasma membrane of myelin-forming Schwann cells in the peripheral nervous system (PNS) or oligodendrocytes in the central nervous system (CNS), around the target axon. After a few turns, cytoplasm is extruded by the apposition of the cytoplasmic surfaces of the membrane to allow compaction of the mature multilamellar sheath. Under the electron microscope, myelin is characterized by the appearance of alternating periodic lines – the major dense line represents apposition of intracellular membrane leaflets and the double, intraperiod line represents apposition of extracellular membrane leaflets (Raine, 1984) (Fig.1). X-ray diffraction studies also define the distance between one major dense line and the next (or one intraperiod line and the next) as around 170 Å in the PNS and 150 Å in the CNS (Kirschner *et al.*, 1984).

Myelin is laid down in segments along the selected axon. Each segment is called an internode. The axon-bare region between two internodes is known as the node of Ranvier. The integrity of the ensheathment and the compaction of myelin is essential for rapid conduction of the nerve impulse along the axon, since action potentials can only be build up at the nodes which forces the current to jump from one node to the next in a "saltatory" fashion. This mode of conduction is ten times faster than conduction along unmyelinated axons.

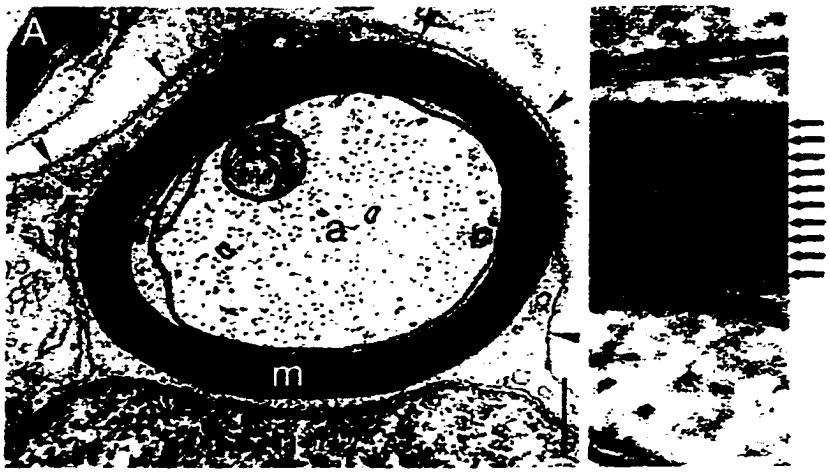



Fig.1: Electron micrograph of a cross section of a myelinated axon from a 5-day-old mouse sciatic nerve (copied from Scherer, Neuron 1997 Jan;18(1):13-6). The axon is surrounded by the Schwann cells that produced the myelin sheath. (A) An axon (a), its myelin sheath (m), Schwann cell nucleus (n), and basal lamina (arrowheads). Scale bar, 0.5 mm. (B) Compact myelin is composed of alternating major dense lines (arrows) and interperiod lines; the distance between the major dense lines is 17 nm.

The proper functioning of myelin is important. Demyelination will impair transmission of nerve impulses and adversely affect nerve communication. Several human neurological diseases resulting from deficiency or disruption of myelin have been identified, such as multiple sclerosis in the CNS and Guillain-Barre Syndrome (GBS) in the PNS. Genetic or environmental factors such as virus infection, trauma, or necrosis, all are able to cause damage to the myelin (see review, Raine, 1984). Unfortunately, most demyelinating diseases are still mysterious and not yet curable. Therefore, understanding myelin and myelination at the molecular and cellular levels may be helpful in the prevention and treatment of demyelinating diseases.

**1.2. Myelin composition**

Like any other membranes, myelin is composed of lipids and proteins except that its lipid content is much higher than usual plasma membranes. Lipids, including cholesterol (the most abundant), glycolipids and phospholipids, comprise 70%-80% of dry weight of the myelin (Rumsby, 1978). This abundance, together with the highly compacted, multilayer membranes, ensures myelin's role as an insulator of nerve fibers. As are lipids in other membranes, the distribution of various myelin lipids in the membrane bilayer is asymmetrical, and tends to change during myelin extension and assembly (see review, Brophy, 1992). In mature myelin, glycolipids are presented more in the outer leaflet, while phospholipids enrich the inner leaflet (see review, Stoffel and Bosio, 1997). Ion-impermeable lipids not only provide the myelin insulative properties, but may also function in the stabilization of the myelin. Mice deficient in galactocerebroside (GalC) and its sulfated derivative, sulfatide (sGalC), were generated by knocking out the *cgt* gene, which encoded UDP-galactose:ceramide galactosyltransferase, a key enzyme in GalC synthesis (Coetzee *et al.*, 1996; Bosio *et al.*, 1996). These $cgt^{-/-}$ mutant mice were able to form compact myelin, nevertheless, the velocity of nerve conduction was dramatically reduced. Moreover, with age, progressive myelin splitting in the ventral column of the spinal cord (Coetzee *et al.*, 1996), a dysmyelinosis with whole-body shivering, seizures and increasing loss of locomotor activity, or reduced growth and death from postnatal day (PND) 20 onwards, developed (Bosio *et al.*, 1996). It was observed that another glycolipid, glucocerobroside (GlcC) was up-regulated in these animals, which is likely to substitute GalC and sGalC in

compaction of myelin (Coetzee *et al.*, 1996). However, a role for GalC and sGalC in myelin function and stability and of vital importance could not be compensated.

The difference in lipid composition between PNS and CNS myelin are minor while difference in PNS and CNS myelin protein composition are more pronounced. Some proteins are found only in CNS or PNS myelin while others are found in both. Po is the major protein exclusively found in PNS myelin and accounts for more than 50% of the total myelin protein. In contrast, proteolipid protein (PLP), and its alternatively spliced isoform DM20, is mainly found in the CNS. PLP (30 kDa)/DM20 (26.5 kDa) is an integral "four-helix-span" membrane protein in a family which includes receptors and ion-channel proteins. Because of the abundance of this protein, it is suggested that PLP is involved in myelin compaction via maintenance of the intraperiod line of CNS myelin (Duncan *et al.*, 1987; Boison *et al.*, 1995). PLP mutations, including missense mutations, deletions and duplications, are responsible for the Pelizaeus-Merzbacher disease (PMD) and spastic paraplegia (SPG) in man or similar dysmyelinating disorders in a range of animal species. Detailed analyses of naturally occurring or genetically engineered mice have provided valuable information about the function of PLP. *Jimpy* mouse, with a single amino acid mutation of PLP protein (Macklin *et al.*, 1987; Nave *et al.*, 1987), displays abnormal myelin compaction (Lees and Brostoff, 1984) and premature death of oligodendrocytes (Knapp *et al.*, 1986; Vermeesch *et al.*, 1990). Over-expression of the wild type *PLP* by two- to four-fold in mice also causes the hypomyelination and glial cell degeneration. In addition, the severity of the defects is dependent on the extent of PLP overexpression (Kagawa *et al.*, 1994). Paradoxically, the $PLP^{-/-}$ null mouse assembles and maintains normal amounts of myelin, at least for one year, but then develops a

progressive myelin pathology (Klugmann *et al.*, 1997). Similar relatively mild symptoms are also apparent in PMD families with a *PLP* null allele (Raskind, *et al.*, 1991), compared to a more severe phenotype associated with the other PLP mutations (Hodes *et al.*, 1993; Ellis and malcolm, 1994). Therefore, absence of PLP/DM20 is probably better than PLP/DM20 mutated with a conservative amino acid substitution or a slight overexpression by gene duplication. However, the functions of PLP are not yet clear.

Another major myelin-specific protein, myelin basic protein (MBP), accounts for about 30% of the protein in CNS myelin and 5%-15% of the total protein in PNS myelin. MBP refers to a group of small positively charged molecules, ranging in size from 14 kDa to 21 kDa (derived by alternative splicing of mRNA). MBP is one of only a couple of myelin proteins which locate wholly intracellularlly, but are still membrane-associated, presumably by electrostatic interactions with the negatively charged head groups of the lipid bilayer. The function of MBP is best demonstrated in the spontaneous mutant *shiverer* mouse where none of five MBP isoforms is synthesized due to deletion of part of the *MBP* gene (Privat *et al.*, 1979; Kirschner and Ganser, 1980). In the CNS of the *shiverer* mouse, the major dense line (MDL) is absent from little compacted myelin. By re-introduction of increasing levels of the wild-type MBP gene into the *shiverer* mouse, both myelin assembly and formation of the MDL are gradually restored (Readhead *et al.*, 1987; Kimura *et al.*, 1989). MBP, therefore, is responsible for compaction and maintenance of the major dense line at least in the CNS.

Other myelin-specific proteins are minor components of the sheath and include myelin-associated glycoprotein (MAG), peripheral myelin protein 22 kDa (PMP22), the enzyme 2', 3' cyclic nucleotide 3' phosphodiesterase (CNP), and basic protein P2, which

are all detected in the PNS. Of particular interest is MAG, which is a member of the immunoglobulin (Ig)-superfamily. Unlike the major myelin proteins, MAG is restricted to noncompacted myelin regions. In both the CNS and the PNS, MAG is expressed at the periaxonal membrane of myelin (Sternberger *et al.*, 1979; Trapp *et al.*, 1984). It has been suggested that MAG plays a role in the initiation of myelination and maintenance of mature myelin (Martini and Schachner, 1986; Trapp, 1990). This view is supported by the *in vitro* studies that myelination is enhanced in cocultures of sensory neurons with transfected Schwann cells that overexpress MAG (Owens *et al.*, 1990), meanwhile, Schwann cells that express MAG antisense RNA fail to form myelin (Owens and Bunge, 1991). Surprisingly, MAG-deficient mice showed near-normal myelination in early adulthood. Only after 8 months old, axons and myelin degenerated in these animals (Li *et al.*, 1994; Montag *et al.*, 1994). This indicates that either MAG is not essential in the initiation of myelin formation *in vivo*, or alternatively, there is sufficient redundancy in function that some other molecules substitute for MAG in its absence. However, MAG's role in glia-neuron interactions has never been weakened. MAG has been shown *in vitro* as a bifunctional molecule, either in promoting (Schneider-Schaulis *et al.*, 1991) or inhibition (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994) of neurite outgrowth, depending on the neuronal cell type and its developmental stage. Furthermore, MAG may participate in regulation of axonal caliber. It was shown that loss of MAG by MAG-deficient mice resulted in reduced axonal caliber, neurofilament spacing and neurofilament (NF) phosphorylation (Yin *et al.*, 1998). This is in support of the hypothesis that, MAG interacts with its putative receptor on the axonal membrane, actives a neuronal kinase or inhibits a phosphatase. Changes in NF phosphorylation

might then alter NF packing density, and therefore axonal caliber (de Waegh *et al.*, 1992). In the PNS, MAG is also present in the outermost layer of the sheath, paranodal loops and Schmidt-Lanterman incisures, which are all areas where Schwann cell cytoplasm is found and connected. Whether MAG has an additional function related to these locations has to be determined.

Another minor PNS myelin protein, PMP22 is the most recent to be identified. PMP22 is synthesized largely by Schwann cells and localized mainly to compacted myelin. Like PLP, a four-transmembrane domain protein, PMP22's sequence is homologous to the murine growth arrest-specific (*gas-3*) gene (Manfioletti *et al.*, 1990) and it is differentially regulated during development, nerve injury and nerve regeneration (Snipes *et al*, 1992; Kuhn *et al*, 1993). *Trembler* mutant mice, characterized by severe PNS hypomyelination and increased Schwann cell proliferation (see review, Nave, 1994; Suter *et al.*, 1993), carry a point mutation in PMP22 (Suter *et al.*, 1992). This implies that PMP22 is critical for Schwann cell proliferation and differentiation. In humans, mutations in PMP22 are associated with one subtype of the demyelinating Charcot-Marie-Tooth disease. Another subtype of CMT is linked to mutations in Po.

Examined under the electron microscope, myelin in the PNS and CNS is morphologically very similar to each other. However, there are some differences between the two. PNS myelin contains Schmidt-Lanterman incisures, which are funnel-shaped domains of noncompact myelin that traverse the compact myelin sheath and are filled with Schwann cell cytoplasm (Raine, 1984). Proteins in the membranes of incisures are different from compact myelin, and are mostly molecules that form tight junctions, adherens junctions, and gap junction. E-cadherin and connexin 32 (Cx32) are

found at this area and also in the paranodes. Cx32 is a gap junction protein with four transmembrane domains. Six Cx32 molecules cluster in the plasma membrane to form a hemichannel. Hemichannels in the cell membranes of adjacent cells form functional channels that allow ions and small nutrients to diffuse directly across the myelin sheath, which is a thousandfold shorter distance than the circumferential pathway through the Schwann cell cytoplasm. The importance of this pathway is realized by the finding that mutations in Cx32 cause the X-linked form of Charcot-Marie-Tooth disease (Bergoffen *et al.*, 1993).

### 1.3. Myelin formation

Schwann cells and oligodendrocytes are responsible for myelination in the PNS and the CNS, respectively (Raine, 1984). A single Schwann cell only myelinates a single axonal segment while a single oligodendrocyte can elaborate up to 50 independent myelin sheaths around different axons. Phylogenetically, this is an evolutionary advantage to have a CNS myelinating cell over a PNS myelinating cell as it saves space by enabling a single cell body to control the myelination of several internodal segments (see review, Colman *et al.*, 1995).

During the development of the PNS, Schwann cell precursors, first identified at embryonic day 12 (E12) in the major peripheral nerve trunks of mice, emigrate from the neural crest, contact and migrate along the surface of previously extended bundles of axons, and undergo a series of events (see review, Mirsky and Jessen, 1996;1998) (Fig.2).

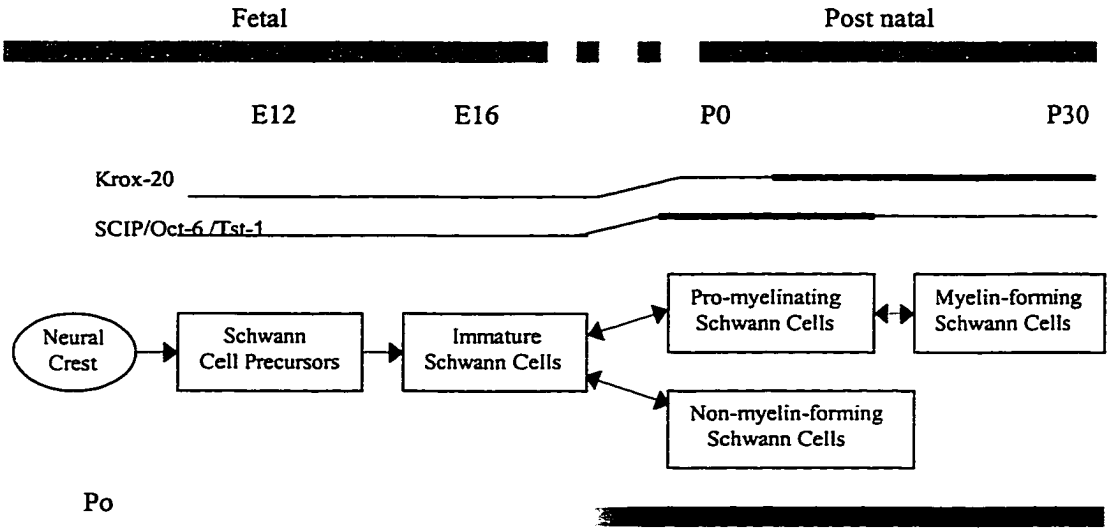



Fig.2: Schematic depiction of mouse Schwann cells development and de-differentiation. The time course for each developmental stage is given. The expression profile of the SCIP and Krox-20 transcriptional factors and Po is indicated by lines or bar. The density of line or bar represents the relative expression levels (adapted from Arroyo *et al.*, J Neurosci. 1998 Oct 1;18(19):7891-902).

Schwann cell cytoplasmic processes first penetrate axon bundles, and begin to segregate axons into large families of clustered fibers. Along with immature Schwann cell proliferation and coupled neuronal death and axon loss, this segregation process continues and results in progressively smaller axon groups, until a one to one relationship between axons and Schwann cells is achieved. At this point, Schwann cells deposit a basal lamina, upregulate expression of myelin-specific genes and start to expand their plasma membranes. Once two to six loose wraps of cytoplasm are elaborated, compaction begins (Peter and Muir, 1959; Webster, 1971; Webster and Favella, 1984; Peters *et al.*, 1991).

However, becoming myelin-forming Schwann cells is not the only destiny for Schwann cell precursors. Some of them acquire the nonmyelin-forming phenotype upon maturation. Nonmyelin-forming Schwann cells are usually associated with more than one axons and express a specific set of markers, including the low-affinity NGF receptor (NGFR), the growth-associated protein 43 (GAP-43) and the cell-adhesion molecules N-CAM and L1, but not express myelin-specific proteins (Jessen and Mirsky, 1992). It is believed that the axon-derived signals determine the phenotype of mature Schwann cells and myelin-forming and nonmyelin-forming Schwann cells seem essentially inter-convertible. Given the right conditions, both myelin-forming and nonmyelin-forming Schwann cells lose their differentiated phenotype and revert to a cell type similar to immature Schwann cells. This phenotypic regression occurs *in vivo* during nerve degeneration (see review, Scherer and Salzer, 1996) or *in vitro* by culturing Schwann cells in serum-containing medium in the absence of axonal contact (Mirsky *et al.*, 1980). Upon re-establishing contact with axons, de-differentiated cells once again adopt a differentiated phenotype (Weinberg and Spencer, 1975, 1976; Aguayo *et al.*, 1976; Jessen *et al.*, 1987).

In recent years, some discoveries have been made to reveal the molecular identity of axonal signals in Schwann cell proliferation and differentiation. Some growth factors have been identified, which include neuregulin (NRG), platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), transforming growth factor beta (TGF-β) and insulin-like growth factor. Neuregulins (NRGs) are a family of proteins that are also referred to as glial growth factors (GGFs), heregulins, acetylcholine-receptor inducing

activity (ARIA), or neu differentiation factor (NDF) (see review, Lemke, 1996). They are generated by alternatively splicing from a single gene. These factors belong to the EGF super family and possess an EGF-like domain which can bind to tyrosine kinase receptors of the EGF receptor family, that include Erb-1, -2, -3 and –4. It was shown *in vitro* that NRGs induce the commitment of neural crest cells to progress along the Schwann cell lineage (Shah *et al.*, 1994). In addition, Dong *et al.* (1995) have shown that NRGs mimic the effects of neuron-derived proteins that block programmed cell death of Schwann cell precursors and support the phenotypic transformation of precursor cells into Schwann cells on Schedule *in vitro*. These effects are neutralized if a hybrid protein containing the extracellular domain of the high-affinity NGR receptor ErbB4 is added to the culture. Considering that NRG mRNA is highly expressed by neurons in rat and mouse embryos during the development (Marchionni *et al.*, 1993; Meyer and Birchmeier, 1994; Orr-Urtreger *et al.*, 1993), these findings suggest that axon-derived NGRs may be responsible for survival and progression of Schwann cell lineage. It was shown that transgenic mice in which the neuregulin gene has been knocked out have greatly reduced numbers of precursor cells in peripheral nerves (Meyer and Birchmeier, 1995). NRGs are also potent mitogens for Schwann cells *in vitro* (Minghetti *et al.*, 1996). It was shown that antibodies against NRG or its receptor blocked the axon-driven Schwann cells DNA synthesis *in vitro* (Morrissey *et al.*, 1995). Furthermore, it has been suggested that axon-derived NGRs play a role in matching the number of Schwann cells to the number of nerve fibers by regulating apoptotic cell death of neonatal Schwann cells (Grinspan *et al.*, 1996; Syroid *et al.*, 1996).

On the other hand, members of the TGF-β family antagonize the proliferation-promoting effect of axons on Schwann cells in co-culture (Einheber *et al.*, 1995). TGF-βs also prevent the axon-driven differentiation of Schwann cells toward myelin production (Einheber et al., 1995; Guénard *et al.*, 1995). Application of TGF-β to cultured Schwann cells was shown to up-regulate NCAM and L1 expression (Stewart *et al.*, 1995). Taken together, these results suggest that TGT-βs might play a role in generating the nonmyelin-forming Schwann cells in developing nerves (Mews and Meyer, 1993; Einheber *et al.*, 1995; Guénard *et al.*, 1995), or alternatively, act earlier in development to maintain the pre-myelin phenotype, thereby indirectly regulating the onset of myelination (Morgan *et al.*, 1994; Einheber *et al.*, 1995; Stewart *et al.*, 1995).

The formation of the basal lamina by Schwann cells is a prerequisite for myelination. Under conditions that prevent basal lamina deposition in co-cultures, Schwann cells still express myelin-specific genes (Owens and Bunge, 1989; Brunden and Brown, 1990), but fail to ensheath and myelinate the nerves (Bunge *et al.*, 1986). It has been suggested that neurons play an important role in the regulation of the basal lamina assembly (Carey and Bunge, 1981). Integrins, the major class of extracellular matrix (EMC) receptors, have been implicated in the myelination process. Axonal contact induces a switch in integrins from α6β1 to α6β4 in Schwann cells (Einheber *et al.*, 1993; Feltri *et al.*, 1994; Niessen *et al.*, 1994). Further, Schwann cell myelination of DRG neurons is inhibited by anti-β1 integrin antibodies *in vitro* (Fernandz-Valle *et al.*, 1994).

Despite the rapid-growing information on the functions of peripheral neurons in Schwann cell proliferation and differentiation, little is known about the role of peripheral

axons in controlling the onset of myelination. The observation that small axons (<1μm in diameter) are almost never myelinated suggests that the diameter of the axon is critical for the initiation of myelin formation (Friede, 1972). Furthermore, the internodal length and the thickness of myelin sheath are proportional to axon caliber (Friede and Samorajski, 1967; Friede *et al.*, 1982; Voyvodic, 1989). However, whether the size of an axon can be related to molecular changes occurring on the surface of the axon membrane, and it is the these changes that initiate myelination has to be investigated.

Persistent axonal contact is also required for myelin maintenance (LeBlanc and Poduslo, 1990; see review, Mirsky and Jessen, 1988; Scherer and Salzer, 1996). In Wallerian degeneration, where axons degenerate due to distal nerve transection, the associated myelin-forming Schwann cell undergoes dedifferentiation to a pre-myelinating state in respond to the axon loss and the expression of myelin-specific proteins are down-regulated. When axons regenerate, normal differentiation and expression of myelin genes are recapitulated. The signaling mechanisms in the Schwann cell controlled by the presence of axons are not well understood. Cyclic AMP (cAMP) has been suggested as such a mediator in the signaling progress between axons and Schwann cells *in vivo* (Lemke and Chao, 1988; Morgan *et al.*, 1991; Kelly *et al.*, 1992; Porter *et al.*, 1986; Sobue and Pleasure, 1984; Sobue *et al.*, 1986). It was shown that Schwann cell isolated from neonatal rats, when exposed to cAMP elevating agents such as forskolin, had up-regulated myelin gene expression (Lemke and Chao, 1988; Morgan *et al.*, 1991; Sobue and Pleasure, 1984; Sobue *et al.*, 1986), and transformed to a flat shape (Sobue *et al.*, 1986; Morgan *et al.*, 1991). These responses mimic some aspects of axonal contact. The

transcription factor CREB (cAMP responsible element binding protein) that translates an increased intracellular cAMP concentration into an altered expression of appropriate target genes, is present in Schwann cell throughout development (Stewart, 1995). However, no CREB-cAMP responsive elements have been found in any of the myelin genes (Zhang and Miskimins, 1993; lemke *et al.*, 1988; Roesler *et al.*, 1988). Furthermore, the effect of cAMP on myelin gene expression in cultured Schwann cells requires extended incubation in cAMP elevating agents. The up-regulation of myelin gene expression in response to cAMP required 36 hours (Lemke and Chao, 1988) to 3 days (Morgan *et al.*, 1991). In contrast, genes with a cAMP responsive element or genes which require protein synthesis in response to cAMP can be up-regulated within minutes to hours of cAMP stimulation (Rickles *et al.*, 1989; Yamamoto *et al.*, 1990; Sasaki *et al.*, 1984). Therefore, a direct link between the myelin genes and cAMP seems unlikely.

In addition to CREB, several other transcription factors are present in Schwann cells as well (Blanchard *et al.*, 1996; Topilko *et al.*, 1996; Zorick and Lemke, 1996; Scherer, 1997). Among them, Krox-20 and SCIP (suppressed cAMP-inducible POU protein)/Oct-6/Tst-1 are the unrelated two modulating the maturation of myelinating Schwann cells. Inactivating the zinc finger gene *Krox-20* by targeted, in-frame insertion of the LacZ gene prevents myelination in the mouse PNS. Schwann cells in Krox-$20^{-/-}$ mutant mouse only initiate axon ensheathment but fail to up-regulate the major myelin proteins such as Po and MBP. They seem to be permanently arrested at the pro-myelinating state (Topilko *et al.*, 1994). In addition, it was shown that up-regulation of Krox-20 during development and in regenerating nerves coincides with the high level of expression of Po during myelination, and its expression still maintains high in adult

nerves (Blanchard *et al.*, 1996; Zorick *et al.*, 1996). However, it is uncertain if Krox-20 activates the transcription of myelin genes directly, as the target sites for this zinc finger protein in the corresponding promoter regions have not been yet identified (Topilko *et al.*, 1994).

Compared to Krox-20, the POU transcription factor SCIP/Oct-6/Tst-1 has a different temporal profile of expression. Its mRNA is transiently expressed with a peak among postnatal day 1 and subsequently down-regulated in myelinating Schwann cells (Monuki *et al.*, 1989; Scherer *et al.*, 1994). In *SCIP/Oct-6/Tst-1*-null mice, Schwann cells are temporarily arrested at the pro-myelinating stage although the levels of myelin-related mRNA in neonatal *SCIP/Oct-6/Tst-1*-null mice are near normal (Bermingham *et al.*, 1996; Jaegle *et al.*, 1996). This indicates that SCIP/Oct-6/Tst-1 is required by Schwann cells in order to proceed to the myelin-forming phenotype. However, earlier findings about SCIP/Oct-6/Tst-1 are contradictory to the observations in *SCIP/Oct-6/Tst-1*-null mice. It was found that SCIP was able to repress transcription from the MBP and Po promoters *in vitro* (Monuki *et al.*, 1990, 1993; He *et al.*, 1991). Mice that express a truncated, dominant negative form of SCIP under the control of the Po promoter, exhibited premature myelination during postnatal development, hypermyelination in adulthood, and an overall elevated expression of myelin genes (Weinstein *et al.*, 1995). Therefore, SCIP/Oct-6/Tst-1 was proposed as a transcriptional repressor of the late myelin genes in the PNS *in vivo*. This contradiction may be explained by the different genetic manipulations in these experiments. However, since a low level of expression of SCIP/Oct-6/Tst-1 is also found in Schwann cell precursors, as well as in immature, myelinating, nonmyelinating and denervated Schwann cells (Blanchard *et al.*, 1996;

Zorick *et al.*, 1996), it is possible that SCIP/Oct-6/Tst-1 has two separate functions: a positive role in early development, and later a role as a negative regulator of myelination.

On the other hand, myelin-forming Schwann cells can influence axons by stimulating maturing axons to increase their caliber (Aguayo *et al.*, 1977; Pannese *et al.*, 1988; Windebank *et al.*, 1985) and to organize integral membrane proteins, including specific ion channels and pumps, to the node of Ranvier (Waxman and Ritchie, 1985; Black *et al.*, 1990). In mice defected in the Schwann cell components such as PMP22, MAG and Po, axonal properties are significantly changed (De Waegh and Brady, 1990; De Waegh *et al.*, 1992; Fruttiger *et al.*, 1995; Carenini *et al.*, 1997; Yin *et al.*, 1998; Martini *et al.*, 1995b; Frei *et al.*, 1999). Therefore, the axon-Schwann cell interaction is reciprocal and more to be explored.

## 2. Myelin protein Po

### 2.1. Gene and Structure of Po

Po was first identified as the most abundant protein in purified PNS myelin, accounting for more than half the total myelin protein detected on polyacrylamide gels. With an estimated molecular weight of 28,000 ~ 30, 000, Po is a small molecule which is modified post-translationally in several ways, including glycosylation, acylation, and phosphorylation.

Po is also a highly conserved protein across species. The cDNA of Po has been cloned from rat (Lemke and Axel, 1985), mouse (Lemke, 1988), shark (Saavedra *et al.*, 1989), chicken (Barbu, 1990) and human (Hayasaka *et al.*, 1991). The amino acid sequence deduced from their cDNA sequence, together with the bovine Po sequence determined directly by protein chemical methods (Ishaque *et al.*, 1980), revealed that the primary sequence of all identified Po's shared more than 55% homology. Rat Po and human Po are even 94% identical. On the basis of hydrophobicity plots of rat cDNA, it is predicted that, preceded by a 29 amino acid signal peptide, mature Po contains 219 amino acids with a single extracellular (124 amino acid), a single transmembrane (26 amino acid) and a single cytoplasmic (69 amino acid) domains (Lemke and Axel, 1985). This topological disposition was later verified by biochemical studies of Po (D'Urso *et al.*, 1990). Using *in vitro* translation system, it was shown that synthesized Po was presented as a precursor form, containing an extra 29 amino acid sequence at N-terminus compared to the mature form. Cleavage of this signal peptide was signal-recognition-particle (SRP)-dependent and a consequence of insertion of Po into the lumen of rough endoplasmic reticulum (RER). A monoclonal antibody raised against the peptide sequence immediately following the putative membrane-spanning domain of Po, stained permeabilized cells but not nonpermeabilized cells, which further confirmed the membrane topology of Po (D'Urso *et al.*, 1990).

It is particularly interesting that the ectodomain of Po (Poex) resembles a single immunoglobulin (Ig) domain which places Po in a very large group of immunoglobulin-related proteins called the Ig superfamily. Many cell recognition/adhesion molecules like neural cell adhesion molecule (NCAM), MAG, and L1 (Hemperly *et al.*, 1986; Salzer *et*

*al.*, 1987; Moos *et al.*, 1988; Williams and Barclay, 1988) are included in this family. Containing one or more Ig-like domains, these molecules are involved in either homophilic (to itself) or heterophilic (between different molecules) binding reactions. Hence, it is reasonable to predict that Po may function as an adhesion molecule to form the intraperiod line via interactions of its Ig-like domain. The secondary and tertiary structures of Poex, first predicted by Chou and Fasman's computerized modeling (Uyemura *et al.*, 1987) and then by comparison with other known, crystallized structures of Ig molecules (Williams and Barclay, 1988; Wells *et al.*, 1993), were recently confirmed by X-ray crystallographic analysis of Poex (Shapiro *et al.*, 1996). It is suggested that the entire extracellular domain of Po consists of ten anti-parallel β-strands designed as A, A′, B, C, C′, C″, D, E, F, G. They are folded into two β-sheets with D, E, B, A making up one sheet and A′, G, F, C, C′ and C″ making up the other β-sheet. The existence of extra β-strands, C′ and C″, sub-classifies this ectodomain as a V-like Ig domain because it more closely resembles the variable regions (V) than the constant regions (C) of immunoglobulins. As with other Ig-like domains, the two β-sheets are stabilized by hydrophobic interactions between the side chains of antiparallel β-strands, and particularly by disulfide bonds, which for Po is between Cys#21 in β-strand B and Cys#98 in β-strand F. It was demonstrated in our laboratory that if the disulfide bond was disrupted by mutating Cys21 to Ala the adhesive function of Po was lost (Zhang and Filbin, 1994; 1998).

The gene encoding Po protein is a single-copy gene. In human, the 7-kb gene maps to chromosome position 1q22-23 and is split into six exons by five introns

(Hayasaka *et al.*, 1993c; Pham Dinh *et al.*, 1993). Poex, unlike most Ig domains which are encoded by a single exon, is among a few Ig domains encoded by two exons. The intervening intron divides the single Ig domain into two symmetric half domains that are similar in both sequence and length. It is postulated that the contemporary Ig domain may have arisen from an ancestral half-domain that evolved through duplication and subsequent joining (Lemke, 1988; Lemke *et al.*, 1988; Salzer and Colman, 1989). As the smallest and simplest member of the Ig superfamily, Po may be closely related to the primordial form of the whole family.

Po is glycosylated via a single N-linkage at Asn93 within its Ig-like domain (Everly *et al.*, 1973; Kitamura *et al.*, 1979; Ishaque *et al.*, 1980). The oligosaccharide chain contains galactose, mannose, *N*-acetyl glucosamine, and fucose as its core structure and exhibits microheterogeniety in its sulfate and sialic acid content (Uyemura and Kitamura, 1991). However, after axonal injury, this complex-type of carbohydrate may change to a high-mannose type sugar (Poduslo, 1984; 1985; Brunden and Poduslo, 1987). The latter type is often found in Po during the initial stages of myelination (Brunden, 1992). Changes of sugar might alter the binding properties of this myelin protein. It has been demonstrated that the cell-cell adhesion induced by Po is lost completely after the glycosylation site is abolished or after changing the complex sugar form to the high-mannose type (Filbin and Tennekoon, 1991; 1993). It has been suggested that although the N-linked carbohydrates in Po may not be directly involved in Po-mediated cell-cell adhesion (Schneider-Schaulies *et al.*, 1990; Griffith *et al.*, 1992), they may help to maintain the orientation of Po relative to the membrane from which it extends (Filbin and Tennekoon, 1991; 1993). This notion is supported by the crystallographic model of Poex

in that the glycosylation site at Asn93 is directed towards the membrane-proximal base of the molecule (Shapiro *et al.*, 1996; Wells *et al.*, 1993; Inouye and Kirschner, 1991). The extended sugar chain is proposed to ensure an appropriate orientation of the protein at the membrane surface, and also to provide potential sites for interaction with neighboring and apposed Poexs. In addition, HNK 1 (a mouse monoclonal antibody), which recognizes a sulfated glucuronate moiety, reacts with Po in some species (Bollensen and schachner, 1987; Kunemund *et al.*, 1988). The HNK 1 epitope has been shown to be directly involved in the adhesive interactions of many recognition molecules (Ariga *et al.*, 1987; Kunemund *et al.*, 1988). Hence, it may contribute to the binding of Po to Po (Griffith *et al.*, 1992) but may not be considered solely responsible for the adhesion because of its inconsistent expression.

The cytoplasmic domain of Po is highly basic, carrying an overall net charge of +15, a charge comparable to that carried on MBP. MBP is known to mediate the formation of the major dense line in CNS through electrostatic interactions with the negative-charged headgroup of phospholipids bilayer. Since MBP is present at a low concentration in PNS myelin, Po is believed to be its functional counterpart in the PNS (Braun, 1984; Lemke *et al.*, 1988). In support of this notion, a synthetic peptide derived from the cytoplasmic domain of Po has been shown to bind and aggregate negatively charged artificial phospholipid vesicles (Ding and Brunden, 1994). In addition, it has been suggested that the cytoplasmic domain of Po could influence the adhesive function of its extracellular sequences. It was shown in our laboratory that Po truncated in its C-terminal by 59 or 52 amino acids was no longer adhesive (Wong and Filbin, 1994; 1996).

Po is acetylated by palmitic acid, stearic acid and oleic acid at Cys153, which is at the junction of the transmembrane and the cytoplasmic domain of Po (Agrawal *et al.*, 1983; Bizzozero *et al.*, 1994). It is proposed that the fatty acid may interact with the adjacent membrane lipids, help to hold the cytoplasmic sequence of Po in the required conformation for interaction with the underlying cytoskeleton or stabilize the turnover of the protein and prevent lateral diffusion (Bizzozero *et al.*, 1994; Filbin and Tennekoon, 1992). Po is phosphorylated at Ser181, 204, 214 (Brunden and Poduslo, 1987; Suzuki *et al.*, 1990) and as yet unidentified Tyr residues (Iyer *et al.*, 1996). Unlike glycosylation and acetylation, which take place in the endoplasmic reticulum (ER) or Golgi apparatus during protein synthesis, phosphorylation occurs after myelin assembly (Brunden and Poduslo, 1987). Since it has been suggested that the phosphorylation of Po could influence the adhesive capabilities of the cytoplasmic domain (Ding and Brunden, 1994), a proportion of Po molecules could be undergoing adhesion and de-adhesion in compact myelin through phoshorylation/dephosphorylation (Filbin *et al.*, 1996). If this is the case, it provides the possibility of movement of molecules, especially Po, within the seemingly rigid myelin membrane. Phosphorylation may also play a role in myelin formation. It has been shown that rat Po may be phosphorylated at one or more tyrosine residues during postnatal development, and the tyrosine phosphorylation reaches a peak at postnatal day 5 when the formation of myelin is maximal (Iyer *et al.*, 1996). However, the significance of the phosphorylation of the cytoplasmic domain of Po is largely unknown including its possible role in signal transduction.

## 2.2. Function of Po

### 2.2.1. Po in the Compaction of Myelin

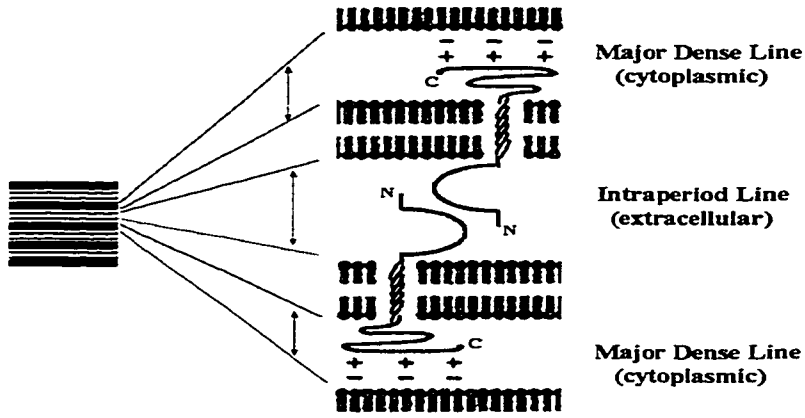



Fig.3: Schematic representation of Po molecules in compaction of PNS myelin. The extracellular domain of Po at the opposing plasma membranes interacts with each other homophilically to form myelin's intraperiod line while the cytoplasmic domain of Po interacts with lipids in the opposing membrane to form the major dense line (modified from *An introduction to molecular neurobiology* edited by Z.W.Hall, Sinauer Associates, Inc).

Because of its abundance and exclusive location to PNS myelin, Po has long been believed to be the major protein responsible for compaction of PNS myelin. It is thought that Po holds the myelin membranes compact at both the intraperiod line via the interactions of its extracellular domain and at the major dense line via the interactions of its cytoplasmic domain with the lipids bilayer (Kirschner and Ganser, 1980; Braun, 1984; Lemke *et al.*, 1988) (Fig.3). Structurally, Po has the characteristics to fulfill both assignments. As stated previously, Po's Ig-like extracellular domain classifies Po to a family known as cell recognition/adhesion molecules. Studies *in vitro* have shown that Po is indeed a homophilic adhesion molecule (D'Urso *et al.*, 1990; Filbin *et al.*, 1990;

Schneider-Schaulies *et al.*, 1990). Meanwhile, the cytoplasmic domain of Po is basic enough to have a heterophilic interaction with acidic lipids of the opposing membrane (Ding and Brunden, 1994; Kischner and Ganser, 1980; Braun, 1984; Lemke, 1988). However, its role in maintaining myelin compact at the major dense line first came from the observation that in the *shiverer* mutant mouse, where no MBP was expressed in either the CNS or the PNS, there was still normal PNS myelin. In contrast, there was an absence of the major dense line in the little compacted CNS myelin that was present (Kirschner and Ganser, 1980). Considering that Po is the most abundant protein found at the major line in the PNS, it is concluded that it compensates for the low levels of MBP in the PNS at this location (Kirschner and Ganser, 1980; Martini *et al.*, 1995b).

The first direct evidence that Po is indeed essential for normal myelination came from retrovirally infected Schwann cells maintained in a culture system. Under normal conditions, when primary Schwann cells are co-cultured with dorsal root ganglion neurons, Schwann cells will differentiate and elaborate compacted myelin sheaths around individual axons. However in these experiments, Schwann cells infected with a retrovirus containing antisense Po RNA (driven by a strong viral promoter) had an impaired ability to form myelin. The extent of failure to myelinate was suggested to be proportionally related to the varying expression level of anti-sense Po RNA (Owens and Boyd, 1991).

In the mouse, the Po gene is located on chromosome 1, but no association with a spontaneous mutation has been reported to date (Kuhn *et al.*, 1990). Morphological examination of peripheral nerves of Po-knockout mice present the most compelling *in vivo* evidence regarding the function of Po. The Po gene in these mice is completely

disrupted by homologous recombination so no Po protein is expressed. In these homozygous, transgenic mice, though a small degree of compaction was observed in some parts of the myelin sheath, most peripheral axons were severely hypomyelinated, and exhibited multiple layers of loose membranes that enwrap axons but without compaction. Furthermore, axons surrounded by the most extensive myelin-like envelopes were degenerated (Giese *et al.*, 1992). At about the same time, genetic linkage analysis in human families revealed the associations between mutations in the Po gene and specific forms of hereditary motor and sensory neuropathies, such as Charcot-Marie-Tooth disease or Dejerine-sottas syndrome (Hayasaka *et al.*, 1993a; Su *et al.*, 1993; Kulkens *et al.*, 1993). This added further support to the view that Po has a crucial role in the integrity of myelin.

Recently, the three-dimensional structure of Po's unglycosylated extracellular domain (Poex) at 1.9 Å was determined (Shapiro *et al.*, 1996). Since it was difficult to crystallize a membrane-spanning protein, only the extracellular portion of Po was crystallized. Oligomerization was seen within the crystal. Based on the computer-aided analysis of this crystal structure, the authors proposed a model that explains how Po molecules bond the myelin membrane together. Po proteins from the same membrane form the tetramer, which is arranged in head-to-tail about fourfold axes. Each tetramer interleaves with four tetramers protruding from the opposing membrane, linking the membranes together. Moreover, the interaction of a Trp side chain of Poex with opposing membrane further stabilizes the compaction. Several pieces of evidence are in favor of this conclusion. First, the protein crystallizes only at a very high protein concentration and at physiologic pH and ionic strength. These findings imply that the

crystallization is driven by the natural properties of the protein not the properties of precipitating agents. Second, analytical ultracentrifugation analysis shows that Poex in solution tetramerizes at different pH and protein concentrations, suggesting the formation of the tetramer is energetically favorable. In addition, high concentration of the Poex solution can reversibly switch between a gel-state and aqueous-state by modulation of temperature. Third, the most convincing information, the dimensions of the model proposed are compatible with some of the known dimensions of native PNS myelin. Within the crystals, layers of molecules are on the order of 46 Å thickness, which is remarkably consistent with the membrane spacing measured from x-ray diffraction patterns of myelin (Kirschner *et al.*, 1989). Two interactions mentioned above, homophilic protein-protein interactions between the tetramers from opposing membranes, and hydrophobic interactions of the side chain of Trp28 from the top of each tetramer with the opposing membrane bilayer as the other, rigidly define that distance. Fourth, the size of a tetramer in the crystal (diameter of about 70 Å) is close to that of a particle found in a freeze-fractured electron micrograph of the myelin membrane (diameter of about 80 Å) (Gabriel *et al.*, 1986). Fifth, the Po and lipid stoichiometry in such network is calculated to be 1:52, which is similar to the 1:86 derived from the chemical composition (Inouye and Kirschner, 1988). Nevertheless, the whole proposal is based on analysis of only the extracellular domain of Po. Whether the tetrameric assembly of whole Po molecules exists in intact myelin is yet to be established. However, more recently, using synchrotron X-ray scattering, tetrameric assembly of full-sequence Po in 0.1% sodium dodecyl sulfate (SDS) solution was reported (Inouye *et al.*, 1999).

### 2.2.2. Po as an adhesion molecule

The recognition that Po is a member of the Ig-superfamily and the availability of its cDNA, allowed examination of the adhesion of Po independently of the myelin membrane by introducing Po cDNA into cells which do not normally express Po. It was shown that Po expression in many cell types conferred cell-cell adhesion and aggregation to those cells (Filbin *et al.*, 1990; D'Urso *et al.*, 1990; Schneider-Schaulies *et al.*, 1990; Yazaki *et al.*, 1992). In our laboratory, Po cDNA was transfected into Chinese Hamster Ovary (CHO) cells and then a single-cell suspension of CHO cells was allowed to adhere. The adhesion was monitored periodically by visualization and by counting the total particle number in the suspension as the incubation proceeded; a drop in total particle number indicated aggregate formation. It was found that the cells expressing Po were at least two orders of magnitude more adhesive than the control transfected cells, not expressing Po protein. Similar results were obtained with Po transfected CV-1 kidney and C6 glioma cells (Schneider-Schaulies *et al.*, 1990; Yazaki *et al.*, 1992). Furthermore, it was demonstrated that Po-expressing cells only adhered to each other rather than to the control cells in a mixed experiment containing both types of cells (Filbin *et al.*, 1990). Therefore, it was concluded that expression of Po induced specific cell-cell adhesion and that the cell adhesion mediated by the extracellular domain of Po was homophilic.

In another experiment, using immunocytochemistry with a polyclonal Po antibody, D'urso *et al* (1990) showed an accumulation of Po molecules at the interface between two intimately contacting Po-expressing HeLa cells. In contrast, the distribution of Po molecules was even over the entire surface when cells were not in contact with each other. Furthermore, an epithelial-like junction was seen along the cell-cell boundaries. It

was suggested that the concentration of Po molecules at opposed surfaces of transfected cells was the result of homotypical interaction between the extracellular domains of Po from opposing membranes and also the inducer of strengthened cell-cell interaction.

Not only Po on cell surfaces induces cell adhesion but as too does an isolated Po extracellular fragment. It was shown that Po's extracellular domain, expressed as a soluble protein in a bacterial expression system, when coated on plastic dishes, was able to bind Po-expressing cell (Schneider-Schaulies *et al.*, 1990) or purified Po protein from human sciatic nerve (Griffith *et al.*, 1992). The binding was specifically inhibited by polyclonal Po antibodies and by adding soluble Po as competitors. These findings further support the suggestion that the backbone of extracellular domain of Po is directly involved in the adhesion and that Po is a homophilic adhesion molecule.

Using a similar system in our laboratory, the precise adhesive domain of Poex was examined (Zhang *et al.*, 1996). Peptides corresponding to three hydrophilic regions of Po's extracellular domain (38-46, 74-82, and 91-95) were generated and used to raise antibodies. It was shown that SDNGT (peptide 91-95) and its antibody completely blocked the adhesion of Po-expressing cells (same results by Yazaki *et al.*, 1992, too). Furthermore, when Asp92 and Gly94 of SDNGT, two highly conserved amino acids found in a subset of Ig-like molecules were mutated, adhesion of Po was lost. What makes SDNGTs special is that the entire sequence is found in the haemagglutinin protein of the 1976 strain of the influenza virus, which when used as a vaccine resulted in an outbreak of the demyelinating disease, Guillian-Barre syndrome (GBS) (Schonberger *et al.*, 1979; Safranek *et al.*, 1991). In addition, the glycosylation site is included in this sequence. By comparison, peptide 74-82 and its antibody inhibited adhesion by 80%,

while neither peptide 38-46 or its antibody had any effect on Po-mediated adhesion. Taken together, it was suggested that the peptide 91-95 may be directly involved in Po's adhesion, while peptide 74-82 may be partially involved. The SDNGT sequence, together with its attached sugar chain, may play a role in holding the molecule in the correct conformation to interact with another *cis* or *trans* Po molecule.

The effect of the cytoplasmic domain of Po on the adhesion of the extracellular domain of the molecule was investigated (Wong and Filbin, 1994; 1996). Po missing the last 52 or 59 residues of the cytoplasmic domain was expressed on the surface of transfected cells but did not mediate cell adhesion. Using the nonionic detergent NP-40, it was shown that 25-30% of the full length Po was associated with the cytoskeleton, indicated by insolubility in NP-40, while only 5-10% of Po with 52 amino acids truncated was insoluble and none of Po truncated by 59 amino acids was insoluble. This suggested that the influence of Po's cytoplasmic domain on its extracellular domain might be mediated by an interaction between the cytoplasmic domain and the cytoskeleton. Furthermore, it was shown that it was microtubules not microfilaments in the cytoskeleton that were involved in the interaction because the microtubule disrupting drug, colchicine, had an effect on Po's adhesive ability but the microfilament disrupting drug, cytochalasin, did not. When truncated Po was coexpressed with full length Po, truncated Po had a dominant-negative effect on adhesion of wild type Po. That is to say, cells coexpressing both full length and truncated Po did not adhere. Using cross-linking reagents, it was shown that when expressed alone full length Po clustered at the cell surface, but when coexpressed with truncated Po, no such clusters were formed. This indicates that the presence of the truncated form of Po prevents the wild type Po from

associating with each other. A model was proposed based on these observations: The cell adhesion mediated by Po involves two steps. The first step is a low-affinity interaction of Po extracellular domains with each other in a *cis*-manner and with the cytoskeleton. The initial interaction of Po extracellular domain in *trans* triggers a change in the interaction of the cytoplasmic domain with the cytoskeleton. In the second step, the cytoskeleton reorganizes and pulls back, inducing Po clustering which in turn strengthens the adhesion of the extracellular domain. The presence of truncated Po would dilute the necessary number of adhesive wild type Pos in each cluster and lower the adhesion affinity per cluster to such an extent that Po loses the ability to hold the two membranes together. The formation of clusters proposed here is in accord with the homotetramer model proposed from the crystal structure of Poex.

### 2.2.3. Other possible roles of Po

In addition to being a structurally adhesive protein in PNS myelin, Po may have a more dynamic role. Studies *in vitro* suggest that Po may promote neurite outgrowth. Coculturing experiments have shown that Po-expressing cells (CV-1 and C6 cells) can enhance the outgrowth of neurites from dorsal root ganglia neurons (Schneider-Schaulies *et al.*, 1990; Yazaki *et al.*, 1991) and cortical neurons (Yazaki *et al.*, 1994). This promotion of neurite outgrowth was specifically inhibited by anti-Po polyclonal (Schneider-Schaulies *et al.*, 1990) or monoclonal (Yazaki *et al.*, 1991) antibodies. Furthermore, when Po-expressing glioma cells were implanted into transected spinal nerves in rat, there was significant axonal elongation into these areas as compared with

control animals (Yazaki *et al.*, 1994). These finding suggest that Po may promote neurite extension through heterotypic interactions with unidentified partners on the neuron (Yazaki *et al.*, 1994). However, the physiological relevance of this capability is questionable. Although some Po mRNA is expressed early in a subset of neural crest precursors (Bhattacharyya *et al.*, 1991; Zhang *et al.*, 1995), the majority of Po is not synthesized and expressed until axons have grown out and made contact with the Schwann cells. Therefore, the neurite-promoting activity of Po may not play a very significant role in normal nervous system development, but rather it may play a role in nerve regeneration and repair after axonal injury and degeneration when the regenerating axon tips would come into contact with Schwann cells and myelin debris expressing Po. Indeed, phylogenetically there is certain correlation between Po expression and the ability of neurons to regenerate. Po protein is expressed in the CNS of fish as well as in the mammalian PNS, where neurons are able to regenerate.

In myelin formation, Po may have some roles other than the compaction and maintenance of myelin. Axon myelination is a multi-step process with a sequence of events including glial cell migration, axon-glial recognition, ensheathment and segregation of axon, membrane spiraling and final compaction. Each event involves up-regulation of some proteins and down-regulation of the others. For instance, N-cadherin may be important for the initial contact of neuron and Schwann cell (Letourneau *et al.*, 1991), L1 for the differentiation of Schwann cells (Seilheimer *et al.*, 1989; Wood *et al.*, 1990), while MAG for the ensheathment of axons (Owens and Bunge, 1991). The orchestration of these events must involve signal transduction. Since Po mRNA is expressed before myelination begins (Martini *et al.*, 1988; Bhattacharyya *et al.*, 1991;

Sherman *et al.*, 1993; Lamperth *et al.*, 1990; Zhang *et al.*, 1995), at a stage when other adhesion molecules such as L1, N-CAM and MAG are present, hence it is possible Po may play some role in the regulation of expression of these molecules. In fact, lack of Po did result in an abnormal expression of many myelin proteins like PLP, L1, N-CAM, MAG and the low affinity NGF receptor in Po $^{-/-}$ mouse (Giese *et al.*, 1992). On the other hand, it was suggested that Po expression might be required for the spiralling of the Schwann cell membrane and progression of myelin formation. It was shown that the disruption of Po both in Po-deficient mouse (Giese *et al.*, 1992) and in anti-sense Po RNA transfected Schwann cells (Owen and Boyd, 1991) caused some Schwann cells arrested in the stage of 1:1 association with large caliber axons.

It is still unknown how the Schwann cell can tell how much myelin lamellae should be laid down in proportion to the diameter of the axon. A signal must be transmitted through layers of myelin to the nucleus of the Schwann cell. Knowing that there is ongoing phosphorylation and dephosphorylation of Po in mature myelin (Brunden and Poduslo, 1987), it is speculated that Po plays a role in determining myelin thickness. Actually, this notion is supported by the observations in mice bred to mis-express Po and/or MBP. It was shown that mice deficient for MBP ($Po^{+/+}/MBP^{-/-}$, *Shiverer*), heterozygous for Po ($Po^{+/-}/MBP^{+/+}$), or heterozygous for both genes ($Po^{+/-}/MBP^{+/-}$) each had normal number of myelin lamellae surrounding axons. In contrast, mice heterozygous for Po and deficient for MBP ($Po^{+/-}/ MBP^{-/-}$) had reduced thickness of myelin around axons (Martini *et al.*, 1995a). This suggests that both Po and MBP contribute to the determination of myelin thickness.

In addition, expression of Po in HeLa cells, a carcinoma cell line originated from cervical epithelium but devoid of its characteristic morphological features, transformed the cells back to an epithelial phenotype, characterized by formation of the epithelial junctions – tight junctions, adherens junctions, and desmosomes (D'Urso *et al.*, 1990; Doyle *et al.*, 1995). This event is associated with upregulation of N-cadherin and desmoplakin. It is proposed that the tight adhesion elicited by Po triggers the signaling pathways involving cadherins and catenins in the carcinoma, which ultimately leads to its reversion to an intact epithelial.

**2.3. Expression and Regulation of Po during development**

Phylogenetically, Po is a very ancient molecule. Its existence could be dated as early as the first real compact myelin is formed in Cartilageneous fish about 440 million years ago. In cartilaginous and bony fish (their contemporary descendants), Po has been identified as the primary mediator of compaction not only in PNS myelin but also in CNS myelin (Waehneldt *et al.*, 1986; Kirschner *et al.*, 1989; Saavedra *et al.*, 1989; Waehneldt, 1990; Stratmann and Jeserich, 1995). PLP, by contrast, is not present until the terrestrial vertebrates (amphilia, reptilia, aves, and mammalia) arose from the evolution, and only then, replaces Po in the compaction of CNS myelin (Franz *et al.*, 1981; Waehneldt *et al.*, 1985). Based on that, it was previously thought that expression of Po and proteolipid proteins is mutually exclusive in the CNS. Studies by Yoshida and Colman (1996), however, revealed that DM20, an isoform of PLP, was detected high in the CNS of bony

fish along with Po. In amphibia, where terrestrial PLP just evolves from DM20, Po was found co-existent with PLP in both CNS and PNS myelin. However, it is still a mystery that how and why Po is completely absent from postamphibian vertebrate CNS and relegated to the PNS in which it plays a critical role in myelin membrane adhesion.

During development, the amount of Po in PNS myelin increases and is well correlated with myelin formation (Wiggins *et al.*, 1975; Wood and Engel, 1976; Uyemura *et al.*, 1979). Po mRNA is readily detectable in rat sciatic nerves at birth when myelination begins, and increases about ten-fold to a peak at postnatal day 14 when myelination and compaction rates are at the highest levels. Then, upon nerve maturation and the completion of myelin compaction, Po levels fall to a steady state in the adult (Lemke and Axel, 1985). Similarly, dramatic increase in Po levels is also observed during the remyelination after axotomy (Lemke and Chao, 1988; LeBlanc and Poduslo, 1990). Previously, it was thought that Po is only expressed in the myelinating Schwann cells as the consequence of axonal contact (Uyemura *et al.*, 1979; Trapp *et al.*, 1981; Politis *et al.*, 1982). Now it is realized that Po is also expressed independently of myelination. For instance, upon nerve transection, Schwann cells undergo complete demyelination but remain basal levels of Po expression (Poduslo *et al.*, 1985). Furthermore, Schwann cells isolated from postnatal day 1 rat sciatic nerves, when cultured *in vitro* in the absence of myelination, constitutively express substantial levels of Po protein (Cheng and Mudge, 1996). Analysis of developing embryos has also revealed the presence of Po long before the commencement of myelination that occurs after birth. Po mRNA is detectable as early as E12 in mouse embryos (Sommer *et al.*, 1995), E4 in chicken embryos, and E14 in rat embryos (Zhang *et al.*, 1995). At these stages, the

transition of Schwann cell precursors to immature Schwann cell phenotype has not even occurred yet (Mirsky and Jessen, 1996). In addition, Po protein is found in rat hind-limb nerves at E18 (Baron *et al.*, 1994) and in Schwann cell precursors isolated from E14 sciatic nerves (Lee *et al.*, 1997). Furthermore, the expression of Po during development is not confined to Schwann cells that will eventually myelinate axons, but has also been detected in non-myelinating glial cells of the PNS, such as satellite cells in mouse sympathetic neurons (Sommer *et al.*, 1995) and trigeminal ganglion in the rat (Lamperth *et al.*, 1989). More strikingly, Po mRNA and protein is found in a subpopulation of migrating neural crest cells in rat (Lee *et al.*, 1997) and chicken (Bhattacharyya *et al.*, 1991) embryos, respectively. Given the earlier expression of Po before the onset of myelination, it is conceivable that Po has additional functions apart from its role in myelin compaction (see earlier title). However, the levels of Po expression at these early developmental stages appear to be considerably lower than that during myelination (Zhang *et al.*, 1995).

Clearly, axons play a role in controlling Po levels during Schwann cell maturation. Positive signals from axons induce high Po expression and myelination during both development and regeneration, and when axons are withdrawn from Schwann cells, Po levels decrease to basal levels (Poduslo *et al.*, 1985; Trapp *et al.*, 1988; see review, Jessen and Mirsky, 1991). However, the nature of these axon-derived signals is still unknown. It has been suggested that cAMP may act as a mediator in this signaling process because elevation of intracellular cAMP levels *in vitro* has similar effects on myelin-gene expression (Lemke and Chao, 1988; Morgan *et al.*, 1991; Kamholz *et al.*, 1992; Mews and Meyer, 1993). However, the Po promoter does not have any known

binding sequence for cAMP-CREB (cAMP resposive element binding protein) (Lemke *et al.*, 1988), it is possible that cAMP may mediate the induction indirectly, by regulation of the other transcription factors that interact with the Po promoter.

However, the up-regulation of Po expression by positive signals form the axons upon myelination was questioned by Cheng and Mudge (1996). They showed that, *in vitro*, a constitutive, relatively high Po level was detected in both premyelinating and myelinating Schwann cells without the addition of any axonal-like signals, provided the culture was serum-free. Hence, they suggested that *in vivo*, the low expression of Po prior to the myelination was due to the inhibitory signals within the nerve, rather than the absence of a positive signal from axons. Indeed, there are factors that inhibit Po expression and may be axon-derived. Fibroblast growth factor-2 (FGF2) (Morgan *et al.*, 1994), TGFβ (Mews and Meyer, 1993), and glial growth factor (GGF) (Cheng and Mudge, 1996) have all been reported to down-regulate Po expression. However, their view was disputed by Lee *et al.* (1997). Lee and colleagues showed that, when early myelin-forming Schwann cells were removed from axons and placed in defined medium without any exposure to serum or other agents known to suppress Po expression, high Po expression fell to the basal level. In addition, it was confirmed that elevated cAMP levels induced the Po expression in Schwann cells that had never been exposed to serum. With regard to the high basal level of Po expression observed by Cheng and co-workers in premyelinating Schwann cells, Lee *et al.* (1997) suggested it could be caused by the high sensitivity of their detection methods. Therefore, it is more likely that the presence of positive signals rather than suppression of inhibitory signals from the axon are

responsible for the up-regulation of Po expression in myelin-forming Schwann cells. Nevertheless, the inhibitory theory could be applied to the down-regulation of pre-existing basal levels of Po in non-myelin-forming Schwann cells. Indeed, it was shown *in vivo* that the loss of axonal contact resulted in an up-regulation of Po mRNA in non-myelin-forming Schwann cells (Lee *et al.*, 1997).

## 3. Po and the demyelinating diseases

### 3.1. Characteristics of the demyelinating diseases

In human, the genetically determined failure to assemble or maintain PNS myelin defines a heterogeneous group of diseases, collectively referred to as hereditary motor and sensory neuropathies (HMSN) (see review, Dyck *et al.*, 1993; Lupski *et al.*, 1993; Suter and Snipes, 1995). HMSN include Charcot-Marie-Tooth disease (CMT), Déjerine-Sottas disease (DSD), hereditary neuropathy with liability to pressure palsies (HNPP), and newly identified congenital hypomyelination (CH) (Warner *et al.*, 1996), each of them with a marked heterogeneity, both pathologically and genetically. The clinical classification of these neuropathies into discrete categories is mostly based on pathologic and clinical evaluations, but sometimes it can be difficult because there can be both clinical and pathologic overlap between these disorders.

CMT is the most common HMSN and also one of the largest of human genetic diseases, affecting approximately one in every 2,500 people (see review, Chance and

Fischbeck, 1994). Affected individuals suffer from progressive atrophy and distal muscle weakness, depressed or absent deep tendon reflexes, and foot deformities. Based on electrophysiological and pathologic studies, CMT has further been classified into two types: type 1 (CMT1), the demyelinating form, characterized by moderate to severe reduction of motor nerve conduction velocities (NCV) (always below 38 m/sec), and on biopsy the presence of onion bulbs formed by a process of de- and remyelinization; or type 2 (CMT2), the neuronal form, characterized by axonal degeneration with normal or slightly reduced motor NCV and few "onion bulb" structures. CMT1 (or HMSN I) is mostly inherited in an autosomal dominant manner with an onset usually in the second decade of life, however, X-linked, recessive, and sporadic cases were also described. Genetic analyses in recent years have linked the CMT1 loci to distinct chromosomes. Three loci for CMT1 were mapped to chromosome 17p11.2-12 (CMT1A) (Vance *et al.*, 1989), chromosome 1q21.2-23 (CMT1B) (Bird *et al.*, 1982), and chromosome Xq13.1 (CMT1X) (Gal *et al.*, 1985). A few autosomal dominant CMT1 families were not linked to either CMT1A or CMT1B indicating that at least a third autosomic CMT1 locus existed (CMT1C) (Chance *et al.*, 1992). Autosomal recessive CMT1 was recently designated as CMT4 (or recessive HMSN) as it appeared to be also genetically heterogeneous. CMT2 (or HMSN II), estimated to occur much less frequently than CMT1, is inherited in an autosomal dominant manner. The linkage of CMT2 to 1p35-36 (CMT2A), 3q13-22 (CMT2B), 7p14 (CMT2D) has been established, genetically distinct from all mapped forms of CMT1. However, the first case of CMT2 being mapped to chromosome 1q21.2-23 was reported recently (Marrosu *et al.*, 1998; De Jonghe *et al.*, 1999).

Of all CMT, 71% is CMT1A associated with a 1.5-Mb tandem duplication on chromosome 17p11.2 which includes the gene encoding the peripheral myelin protein PMP22 (Timmerman *et al.*, 1992). The presence of three intact copies of *PMP22* favors the hypothesis of a gene dosage effect as disease mechanism. Single base pair mutations have also been identified in *PMP22* in CMT1A patients. CMT1X, making 10-20% of CMT, is the second common form of CMT after CMT1A. Male patients are more severely affected than the female. Linkage studies have mapped CMT1X to the proximal long arm of the X chromosome, the same region where the gene encoding the gap junction protein connexin 32 resides (Bergoffen *et al.*, 1993). Single base pair mutations of *Cx32* gene have been shown to associate with CMT1X directly. The less common form, CMT1A, which is linked to the Po gene, will be discussed later.

DSS (or HMSN III) is a severe, infantile and childhood onset, hypertrophic demyelinating neuropathy. The clinical features of DSS overlap with those of severe CMT1. Many patients with DSS appear to represent sporadic cases and are usually thought to result from an autosomal recessive gene. Molecular genetic studies have revealed that DSS may be associated with point mutations in either the Po or the *PMP22* gene (Hayasaka *et al.*, 1993b; Roa *et al.*, 1993), and all mutations are present in the heterozygous state, suggesting that DSS may actually be caused by dominantly acting genetic defects.

HNPP is an autosomal dominant disorder which usually develops as a painless mononeuropathy after minor trauma or compression (Windebank, 1993). Sometimes HNPP patients are misdiagnosed as having CMT1 because of mild overlap of clinical features with CMT1. 86% of the HNPP patients is presented with a 1.5-Mb deletion on

chromosome 17p11.2, which is the reciprocal mutation of the CMT1A duplication (Chance *et al.*, 1993; Marimann *et al.*, 1993). Hence, the *PMP22* gene is deleted in HNPP. It is possible that deletion of the *PMP22* gene and underexpression of this locus hold responsible for the HNPP. Further, in a few nondeleted HNPP patients, point mutations in PMP22 were detected.

CH is characterized clinically by early onset in infancy of hypotonia, areflexia, distal muscle weakness, and very slow NCV (Lyon, 1969; Kennedy *et al.*, 1977; Harati and Butler, 1985). In less severe cases, CH is almost indistinguishable from DSS, if diagnosis is only based on physical examination and electrophysiologic studies. More likely, CH is present with the absence of active myelin breakdown and the paucity of the onion bulbs, while DSS with the presence of demyelination/remyelination and an abundance of well-organized onion bulbs. Unfortunately, little is known about the molecular basis of CH, until recently that a point mutation in the Po gene was identified in the CH patients (Warner *et al.*, 1996).

### 3.2. Involvement of Po in the demyelinating diseases

The signs of de- and re-myelination found in peripheral nerve biopsies of CMT 1B patients suggest that the defect is located in the Schwann cells involved in the formation of myelin. As soon as the human myelin protein zero Po gene (*MPZ*) was mapped to chromosome 1q22-23 in the same region with the CMT1B locus (Hayasaka *et al.*, 1993c), it became a good candidate gene for the CMT1B. By sequence analysis of

the Po gene in affected CMT1B patients, three independent studies (Hayasaka *et al.*, 1993a; Su *et al.*, 1993; Kulkens *et al.*, 1993) identified single point mutations in Po in all pedigrees they studied and the mutations co-segregate with the disease. Therefore, it is concluded that CMT1B is associated with mutations of the myelin Po gene.

To date, 57 distinct *MPZ* mutations have been identified in the HMSN patients (Nelis *et al.*, 1999) (Table 1). There are 36 missense mutations, 3 nonsense mutations, 4 in-frame deletions/insertions, and 10 frame-shift mutations. In one case an 8 bp sequence was replaced by a 5 bp sequence resulting in a double missense mutation combined with a one amino acid deletion (Silander *et al.*, 1996). In another case, three missense mutations were present on the same allele (Warner *et al.*, 1997). In one of two 3'-splice-site mutations, a new putative splice site may be created to have one amino acid replaced by two amino acids (Su *et al.*, 1993). In addition, there are three silent mutations and one polymorphism found in *MPZ*. The polymorphism is an Arg to Leu substitution at codon 215, found in an unaffected relative of a DSS patient (Bort *et al.*, 1997), suggesting it is a nonpathological polymorphism or a recessive mutation. Neither monosomy nor trisomy of the *MPZ* locus has been reported, which is unlike another myelin gene, *PMP22*, whose duplication or deletion counts for most cases of CMT1A/HNPP.

Table1: Mutations and polymorphisms in *MPZ* (adapted from Nelis et al., Human Mutat.1999 (13):11-28)

Codon numbering according to the mature Po protein sequence.

| Nucleotide change | Exon | Codon | Domain | Amino acid change | Phenotype | References |
|---|---|---|---|---|---|---|
| ATC-ATG | 2 | 1 | EC | Ile-Met | CMT1 | Hayasaka *et al.*, 1993e |
| GTT-TTT | 2 | 3 | EC | Val-Phe | CMT1-severe | Haites *et al.*, 1998 |
| ACC-ATC | 2 | 5 | EC | Thr-Ile | CMT1 | Gabreëls-Festen *et al.*, 1996 |
| TCC-TTC | 2 | 15 | EC | Ser-Phe | CMT2 | Marrosu *et al.*, 1998 |
| TCC-TGC | 2 | 25 | EC | Ser-Cys | CMT1-severe | Haites *et al.*, 1998 |
| GTC-TTC | 2 | 29 | EC | Val-Phe | CMT1 | Sorour *et al.*, 1998. |
| ATC-TTC | 2 | 33 | EC | Ile-Phe | CMT1 | Nakagawa *et al.*, 1999 |
| TCC-TGC | 2 | 34 | EC | Ser-Cys | DSS | Hayasaka *et al.*, 1993b |
| TCC-TTC | 2 | 34 | EC | Ser-Phe | CMT1 | Blanquet-Grossard *et al.*, 1995 |
| delTCC | 2 | 34 | EC | del Ser | CMT1 | Kulkens *et al.*, 1993 |
| delTTC | 2 | 35 | EC | del Phe | $CMT1^{htz}_{hmz}$/DSS | Ikegami *et al.*, 1996 |
| TAC-TGC | 2 | 39 | EC | Tyr-Cys | CMT1-severe | Sorour *et al.*, 1998 |
| delG | 2 | 46 | EC | frameshift | CMT1 | Haites *et al.*, 1998 |
| TCG-TTG | 2 | 49 | EC | Ser-Leu | CMT1 (1x severe) | Nelis *et al.*, 1994; Latour *et al.*, 1995; Bort *et al.*, 1997; Haites *et al.*, 1998; Silander *et al.*, 1998 |
| CAC-CGC | 3 | 52 | EC | His-Arg | CMT1-severe | Sorour *et al.*, 1997 |
| TAT-TGT | 3 | 53 | EC | Tyr-Cys | 2 CMT1 / DSS | Himoro *et al.*, 1993; Haites *et al.*, 1998; Silander *et al.*, 1998 |
| 8 bp - 5 bp | 3 | 57-59 | EC | | DSS | Silander *et al.*, 1996 |
| GAC-GAA | 3 | 61 | EC | Asp-Glu | CMT1 | Hayasaka *et al.*, 1993a |
| GGG-GAG | 3 | 64 | EC | Gly-Glu | CMT1 | Ikegami *et al.*, 1997 |
| AAA-GAA | 3 | 67 | EC | Lys-Glu | CMT1 | Hayasaka *et al.*, 1993a |
| CGC-TGC | 3 | 69 | EC | Arg-Cys | 3 DSS / 2 CMT1-severe | Warner *et al.*, 1996; Rouger *et al.*, 1996; Gabreëls-Festen *et al.*, 1996; Bort *et al.*, 1997; Haites *et al.*, 1998; Komiyama *et al.*, 1997 |
| CGC-CAC | 3 | 69 | EC | Arg-His | CMT1 | Rouger *et al.*, 1996; Gabreëls-Festen *et al.*, 1996; Hayasaka *et al.*, 1993d |
| CGC-AGC | 3 | 69 | EC | Arg-Ser | CMT1 | Warner *et al.*, 1996 |
| CGC-CCC | 3 | 69 | EC | Arg-Pro | CMT1 | Rouger *et al.*, 1996 |
| ATC-ACC | 3 | 70 | EC | Ile-Thr | CMT1 | Haites *et al.*, 1998 |
| TGG-TGC | 3 | 72 | EC | Trp-Cys | CMT1 | Latour *et al.*, 1995 |
| delA | 3 | 73 | EC | frameshift | $CMT1^{htz}_{hmz}$/DSS | Warner *et al.*, 1996 |
| ATT-ACT | 3 | 83 | EC | Ile-Thr | CMT1-severe | Sorour *et al.*, 1998; Haites *et al.*, 1998 |
| ATA-ACA + AAC-CAC + GAC-AAC | 3 | 85+ 87+ 99 | EC | Ile-Thr + Asn-His + Asp-Asn | DSS | Warner *et al.*, 1997 |
| ins6 | 3 | 89 | EC | insPhe-Tyr | DSS | Ikegami *et al.*, 1998 |
| AAT-AGT | 3 | 93 | EC | Asn-Ser | CMT1 | Blanquet-Grossard *et al.*, 1996 |
| ACG-ATG | 3 | 95 | EC | Thr-Met | 2 CMT1 / 6 CMT2 | Schiavon *et al.*, 1998; Chapon er al., 1999; De Jonghe *et al.*, 1999 |
| del6 | 3 | 95-96 | EC | delThr-Phe | DSS | Schiavon *et al.*, 1998 |
| TGT-TAT | 3 | 98 | EC | Cys-Tyr | DSS | Fabrizi *et al.* 1999 |
| GAC-GAG | 3 | 99 | EC | Asp-Glu | CMT1 | Haites *et al.*, 1998 |
| AAA-AGA | 3 | 101 | EC | Lys-Arg | 1CMT1 / 3 DSS | Gabreëls-Festen *et al.*, 1996; Tachi *et al.*, 1996 |
| CCT-CTT | 3 | 103 | EC | Pro-Leu | CMT1 | Sorour *et al.*, 1998 |
| GAC-GAA | 3 | 105 | EC | Asp-Glu | CMT1 | Nelis *et al.*, 1994 |
| GAC-AAC | 3 | 105 | EC | Asp-Asn | CMT1 | Nelis *et al.*, 1994 |
| ATA-CTA | 3 | 106 | EC | Ile-Leu | CMT1 | Gabreëls-Festen *et al.*, 1996 |
| ATA-ACA | 3 | 106 | EC | Ile-Thr | CMT1/DSS | Roa *et al.*, 1996; Tyson *et al.*, 1997 |
| GGC-AGC | 3 | 108 | EC | Gly-Ser | CMT1 | Roa *et al.*, 1996 |
| ACG-ATG | 3 | 114 | EC | Thr - Met | CMT1 | Haites *et al.*, 1998 |
| g-c | IVS3 | - | EC | 3'- splice site | CMT1 | Bort *et al.*, 1997 |
| TAC-TAA | 4 | 125 | TM | Tyr-stop | CMT1 | Nelis *et al.*, 1994 |
| GGG-AGG | 4 | 134 | TM | Gly-Arg | CMT1 | Nelis *et al.*, 1996 |
| GGG-AGG | 4 | 138 | TM | Gly-Arg | 2 CMT1 / 1 DSS | Hayasaka *et al.*, 1993; Nelis *et al.*, 1996; Sorour *et al.*, 1998 |

Codon numbering according to the mature Po protein sequence.

| Nucleotide change | Exon | Codon | Domain | Amino acid change | Phenotype | References |
|---|---|---|---|---|---|---|
| G-C + delC | 4 | 137 | TM | frameshift | DSS | Tyson *et al.*, 1997 |
| ins4 | 4 | 143 | TM | frameshift | DSS | Tachi *et al.*, 1998. |
| del4 | 4 | 145 | TM | frameshift | DSS | Warner *et al.*, 1996 |
| TAC-TAG | 4 | 152 | IC | Tyr-stop | CMT1 | Nelis *et al.*, 1994 |
| delG | 4 | 156 | IC | frameshift | CMT1 | Bort *et al.*, 1997 |
| ins17 | 5 | 176 | IC | frameshift | CMT1 | Bort *et al.*, 1997 |
| CAG-TAG | 5 | 186 | IC | Gln-stop | CH | Warner *et al.*, 1996; Mandich *et al.*, 1999 |
| 5 in a 9-bp seq | INT5-EX6 | 187 | IC | Thr-GluArg | CMT1 | Su *et al.*, 1993 |
| insGC | 6 | 192 | IC | frameshift | DSS | Rautenstrauss *et al.*, 1994 |
| insCA | 6 | 197 | IC | frameshift | CMT1 | Haites *et al.*, 1998 |
| del4 | 6 | 204 | IC | frameshift | CMT1-severe | Bellone *et al.*, 1996 |
| Polymorphism | | | | | | |
| GTG-GTA | 3 | 73 | EC | Val-Val | - | Nelis *et al.*, 1994 |
| GGGGGA | 5 | 171 | IC | Gly-Gly | - | Roa *et al.*, 1996; Bort *et al.*, 1997 |
| AGC-AGT | 6 | 199 | IC | Ser-Ser | - | Roa *et al.*, 1996; Bort *et al.*, 1997 |
| CGC-CTC | 6 | 215 | IC | Arg-Leu | - | Bort *et al.*, 1997 |

Compared to the *PMP22* and *Cx32*, mutations in the MPZ have caused a more diversified spectrum of peripheral neuropathies. Of 57 mutations, 36 are associated with CMT1B, 10 with DSS, two with CMT2, and one with CH. Two mutations are presented as CMT1B when only one allele is affected, or as DSS when both alleles are affected. The other 6 mutations were reported as either CMT1B or DSS in several unrelated cases. This ambiguity could be explained by an unclear definition of DSS. However it cannot be excluded that the real phenotypic differences exist between the two cases with the same mutation. In that case, environmental factors could have an influence on the clinical severity.

Of all *MPZ* mutations, 77% are located within the coding sequence of the extracellular domain of the Po protein, indicating the importance of this domain in the normal compaction of the myelin sheath. Codon 34 (Ser) and 69 (Arg) are the ones most sensitive to the mutations because more than two different amino acid substitutions are

associated with each. However, mutations at the other domains of the Po protein may be more lethal, hence not countable.

Most affected individuals with CMT1B/DSS are heterozygous for the mutated *MPZ* and presumably express half the dose of the normal protein and half the dose of mutated Po. How the mutated Po protein affects the function of normal Po is not clear. A 50% decrease in gene dosage may be one mechanism responsible for the disease. As seen in Po-deficient mice, the homozygous mice ($Po^{-/-}$) demonstrated a more severe pathology than the heterozygous mice ($Po^{+/-}$) did (Giese *et al.*, 1992; Martini *et al.*, 1995b). The heterozygous mice ($Po^{+/-}$), which initially appear to have normal myelination, eventually developed a progressive demyelination after 4 months of age (Martini *et al.*, 1995). This suggests that half the dose of Po is inadequate for long-term myelin maintenance but is sufficient for initiation and formation of myelination. This phenotype mimics some milder forms of CMT1B. In these patients, the mutated Po could be unstable, poorly transported or poorly inserted to the Schwann cell membrane, and thus easily subjected to degradation, leaving only one non-mutant allele of Po in myelin. However, the reduced levels of Po cannot explain the more severe phenotypes which have an earlier onset. One explanation is that the presence of the mutated Po protein is directly responsible for the abnormalities observed in the disease. In this situation, the mutated Po protein interferes with the functioning of the normal Po protein in a dominate-negative manner. According to the proposed structural model, normal Pos form homotetramer in the same plane, interacting with another homotetramer from the opposing plane (Shapiro *et al.*, 1996). Two types of Po-Po interactions contribute to this network. One is the fourfold side-by-side interaction between the Po molecules from the

same membrane. This is the basis for the tetrameric association. The other is the twofold side-by-side interaction between the tetramers from the opposing membranes. It is suggested that any mutations that alter the ability of Po to interact with other Po molecules cause more severe phenotypes (Warner *et al.*, 1996; Shapiro *et al.*, 1996).

Although tetramer formation is mainly brought about by the extracellular domain of Po, the cytoplasmic domain of Po also play an important functional role in myelin. 6 out of 10 mutations, which directly or indirectly effect the cytoplasmic domain of the Po protein, are associated with the more severe forms of DSS or CH. They are mostly nonsense or frameshift mutations which generate proteins that are slightly shorter or larger than the normal Po. Since it has been suggested that the highly positively charged Po intracellular domain interacts with the negatively charged phospholipid bilayer to help form the major dense line (Ding and Brunden, 1994), a slight alteration of the charge profile may interfere with the formation and compaction of the major dense line.

## 4. Goals of this thesis work

It has been suggested that mutations in Po can result in either the loss of function of Po due to the degradation of a severely misfolded and/or truncated protein, or the generation of a dominant negative protein, where the aberrant Po protein is not degraded but disrupts the homophilic interactions and possibly tetramer formation of the non-

mutant allele of Po (Shapiro *et al.*, 1996; Warner *et al.*, 1996). Site-directed mutagenesis of Po and subsequent biochemical analysis of the resulting proteins would help to determine the exact effects each mutation has on the structure, processing, and homophilic interactions of Po. In our laboratory, we have shown that Po is a homophilic adhesion molecule using the *in vitro* transfection/adhesion assay, in which Chinese hamster ovary (CHO) cells, induced to express Po by transfection of the Po cDNA, were more adhesive compared to control transfected CHO cells, not expressing Po. Using the same system, six site-directed Po mutants will be analyzed individually. They will be transfected into the CHO cells, and their adhesive properties compared to the wild type protein. Furthermore, by co-expression of wild type and mutated Po in the same cell, putative dominant-negative effects will be determined.

In summary, there are mainly three aims in this prospect:

1. Four missense and two nonsense mutations of Po identified in CMT1B or DSS patients will be generated individually by site-directed mutagenesis. The mutants will be expressed in the CHO cells. The ability of each mutated protein to reach the cell surface, to be glycosylated, and to behave as an adhesion molecule will be analyzed (Chapter III).

2. To detect whether the Po mutants have a dominant-negative effect on the wild type protein, each mutated and the wild type Po cDNA will be co-transfected into the CHO cells in a 1:1 ratio. The adhesive property of the co-expressors and that of cells expressing only the wild type Po will be compared, and the effect of Po mutants on the adhesion of the wild type protein determined (Chapter IV).

3. How each Po mutation affects its function and whether the adhesiveness of co-expressors is correlated to its phenotype in disease will be discussed (Chapter V).

# Chapter II

# Methods and Materials

**1. Cell maintenance**

*Dihydrofolate reductase (dhfr)*-deficient CHO cells were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10% complete fetal bovine serum (FBS), proline (40 mg/liter), glycine (7.5 mg/liter), thymidine (0.73 mg/liter), L-glutamine (0.29 g/liter), and hypoxanthine (4.1 mg/liter) (*abb.* cDMEM) at 37°C in 7.5% $CO_2$ air atmosphere. After transfection, hypoxanthine was omitted and dialysis FBS was used instead of complete FBS (*abb.* dDMEM). 100 nM $CdCl_2$ was added 24 hours prior to applicable assay to induce protein expression.

The Myc 1-9E10.2 mouse hybridoma cells (American Type Culture Collection) were maintained in RPMI medium 1640 (Gibco) supplemented with 10% complete FBS. To obtain the maximum amount of secreted myc-antibodies, the medium were collected after incubating the cells for 5-7 days without exchanges. The bulk of supernants was aliquoted in 50 ml and frozen down at -80°C. Otherwise, the myc-sepharoseG beads were prepared by incubating 50 ml myc-supernant with 200 µl 10% (wt/vol) protein G sepharose overnight at 4°C with gentle mixing. The beads were spun down, washed with 3xPBS, and resuspended in final volume of 200 µl.

**2. Subcloning of wild type human Po cDNA into a suitable plasmid**

Plasmid SJL (pSJL) is a suitable vector for transfection of *dhfr*$^-$CHO cells. It carries *dhfr* gene so transfected cells no longer need the supply of glycine, thymidine and hypoxanthine. Furthermore, foreign gene cloned into unique XhoI site can be amplified

using the methotreaxate (MTX)/*dhfr* strategy (Alt *et al.*., 1978). Besides, it contains Geneticin (G418) resistance gene, allowing easy selection after transfection, and a metallothionein promoter downstream from the cloning site, which can be activated by heavy metal like $Cd^{2+}$.

Human Po (hPo) cDNA in pUC19 was given as a gift by Dr. Hayasaka. The EcoRI-EcoRI fragment (1.12 kb) containing the entire Po coding sequence was cut, purified, and cloned into pSJL at XhoI site after adding XhoI linkers at both ends. HPo cDNA insert either in 5'-3' or 3'-5' orientation relative to the promoter was obtained and used for transfection of the CHO cell.

**3. Subcloning of point mutated human Po cDNA**

Since the restriction enzyme map of pSJL was unknown, it would be impossible to perform mutagenesis on Po-cDNA cloned in this plasmid. Therefore, hPo cDNA was subcloned into plasmid Bluescript IIKS (pBS) at XhoI site, with an orientation of 3'-5' relative to the colE1 ori.

The first mutation, Ser34 to Cys, was carried out by two-step recombinant polymerase chain reaction (PCR) (Fig.4). A pair of inside primers was mismatched to Po nucleotides #176 - #198 (5'CAGATGACATCTCCTTCACCTGG3') (numbering according to Hayasaki *et al.*, 1991) with codon of 'TCC' for Ser34 replaced by codon of 'TGC' for Cys (primers were named as Ser sense and anti-sense, respectively). Two end primers matched the sequences about 800 base pairs (bps) apart. One targeted to a sequence on vector pBS containing a HindIII cutting site (so named as HindIII primer)

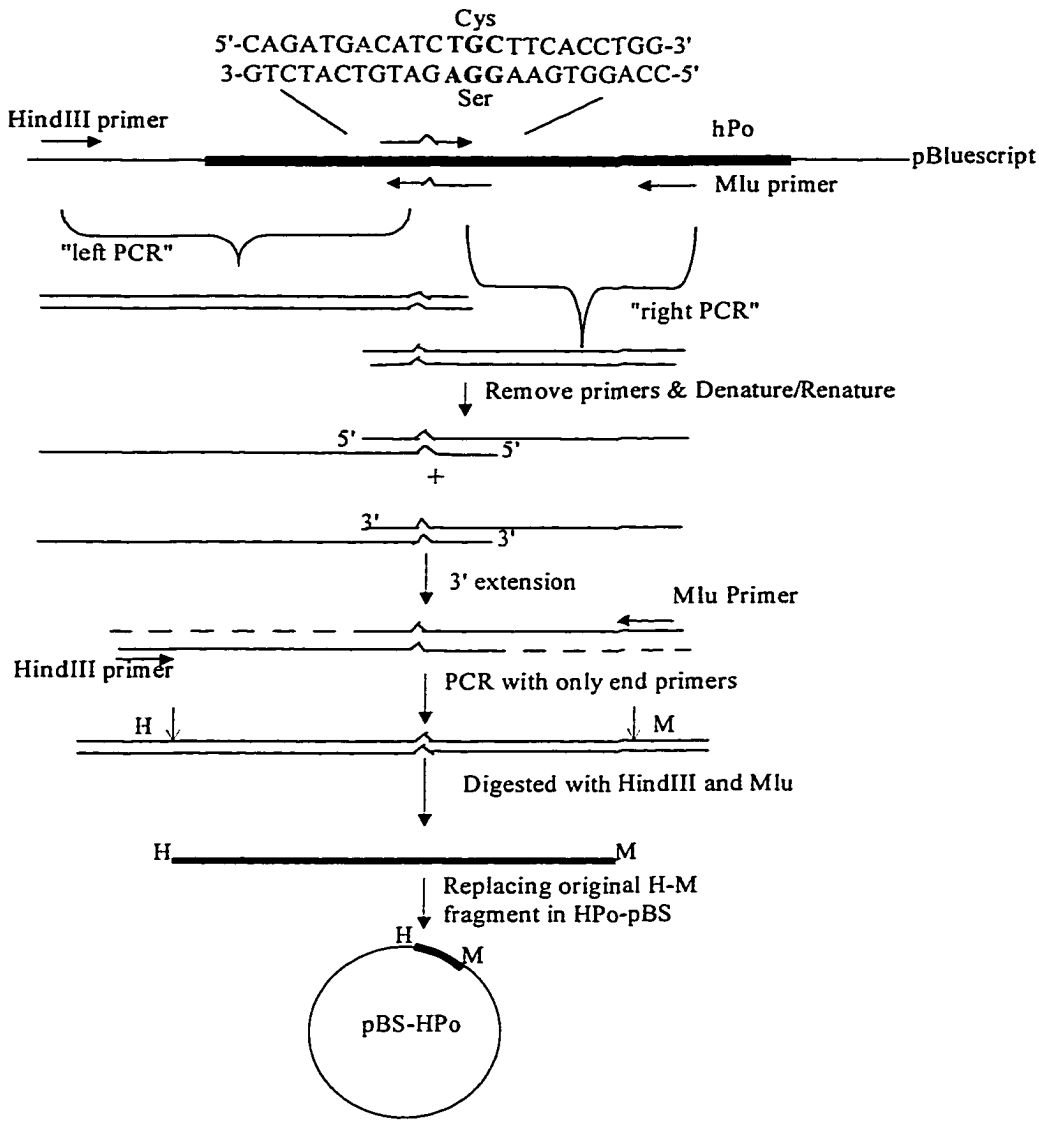



Fig. 4: Schematic diagram of mutation on Po residue Ser34 using recombinant PCR.

and the other matched to the anti-sense Po nucleotides #645 - #667 (3'TCTGCGGTCACGACATACGTTAC5') containing a Mlu cutting site (named as Mlu primer). After primary and secondary PCRs, the 800-bp products were obtained, purified from the gel and digested with HindIII and Mlu. The site-mutated one then replaced the corresponding HindIII-Mlu sequence on original wild type hPo-pBS. The mutation site

was confirmed by dideoxy sequencing (DNA sequencing kit from US Biochemicals), and the mutated Po cDNA was cloned back to pSJL at XhoI site with correct orientation.

Strategene® QuickChange™ site-directed mutagenesis kit was used to mutate all the other five sites individually (Asp61 to Glu, Lys67 to Glu, Tyr125 to stop codon, Tyr152 to stop codon and S34 deletion). For each mutagenesis, a pair of complementary primers, which matches the double-stranded Po sequence but contains the desired mutation, was designed. With the hPo-pBS as template, the primers were extended during the PCR reaction to generate a mutated plasmid with staggered nicks. The parental DNA templates (isolated from *dam*$^+$ *E.Coli* stains) were then digested by enzyme DpnI as they are methylated. The nicked vector DNA incorporating the desired mutation was then transformed into *E.Coli* and amplified. Each mutation was first confirmed by enzyme digestion, as either an additional cutting site was created on the primer (as for Asp61 and Lys67 mutations, and Ser34 deletion), or an original cutting site was abolished by the mutation (as for Tyr125 and Tyr152 mutations), and then by DNA sequencing (facilitated by Hunter College, Dept. Biology). The point-mutated Po cDNA was then subcloned into pSJL.

**4. Transfection/co-transfection**

CHO cells in 80% confluence were transfected with 1μg plasmid DNA per 6-cm dish by calcium phosphate precipitation (Graham and Van der Eb, 1973) followed by a glycerol shock (Frost and Williams, 1978). For co-transfections, the pSJL plasmids containing either myc-tagged wild type rat Po (rPo) (from Dr. Wrabetz in Italy) or

mutated hPo cDNAs were transfected in a 1:1 ratio by the same method. The transfected cells were passed onto 10-cm dish on the following day, when full, passed again to four 10-cm dishes. 400μg/ml G418 (Gibco) was added when cells were 80% confluent. After about 3 weeks, colonies started to appear. At this stage, either a number of colonies were picked, expanded, screened for the Po expression by Western-blot; or for co-transfectants, colonies were pooled, expanded and then subjected to FACS-sorting.

**5. FACS sorting of co-transfectants**

About $10^8$ G418-resistant, co-transfected CHO cells were collected by trypsinization and centrifugation. Cells were first incubated with myc-Ab supernatant (see method 1) for an hour at 37°C, spun down, washed with DMEM three times, and then resuspended in DMEM containing Cy2-conjugated anti-mouse Ig (KPL) (1:250) with a half hour incubation. Then, the cells were washed thoroughly to remove unbound dye and passed through 70μm-diameter cell strainer to eliminate aggregates or fibre in medium. A group of cells with desired brightness were sorted out by the facilities in the medical center of NYU.

**6. Gene amplification**

Usually Po expression was low at initial selection. High level of Po expression was achieved by the *dhfr*/MTX strategy. Cells with multiple copies of the *dhfr* gene were selected by growing the cells in increasing concentrations (0.05-3.0 μM) of MTX. As the

Po cDNA is next to the *dhfr* gene, it will be amplified in the same process. Cells were plated at $10^5$ cells per 10-cm dish, and those surviving after 2-3 weeks at each concentration of MTX were allowed to multiply before being plated on the higher concentration of MTX. At the different stages of amplification, expression of Po protein was monitored by Western-blot. In some cases that high expression of Po was not achieved, single-cell cloning was performed by limiting dilution.

**7. Detection of Po mRNA expression**

Total RNA was isolated from ~$2x10^6$ transfected/co-transfected CHO cells (Qiagen RNeasy mini kit). The RNA was then treated with DNase (Gibco) to avoid any possible contamination of DNA. The first single-chain of cDNA was synthesized by priming with Oligo(dT) with the reverse transcriptase as the catalyst (Clontech Advantage RT-for-PCR kit). The subsequent PCR was carried out by priming with a set of hPo cDNA specific sequences — Ser sense (#176-#198) and Mlu (#645-#667) primers as mentioned above (method 2). Under the stringent condition, the Ser sense primer can only binds to human Po cDNA not rat Po cDNA, probably due to three mismatched nucleotides close to 3'-end. Therefore, for co-transfectants, the above set of primers was used to detect the existence of mutated hPo cDNA. A rat Po cDNA specific primer 5'CCAAGGGTCAACCTTACATCGAT3' (rPo#236-#258), together with Mlu primer, was able to discriminate rPo cDNA from hPo cDNA.

**8. Immunodetection of Po expression by Western blotting**

Cells (80%-90% confluence) were lysed in 1x sodium dodecyl sulfate (SDS) sample buffer containing 62.5 mM Tris-HCl, pH 7.5, 2% SDS, 20 % glycerol and the following proteinase inhibitors: leupeptin, 1 μg/ml; antipain, 2 μg/ml; benzamidine, 10 μg/ml; chemotrysin, 1 μg/ml; pepstatin, 1 μg/ml; and phenylmethylsulfonylfluoride (PMSF), 1 μg/ml. The lysate was homogenized by passage through the needle of 1-ml tubulin syringe and centrifuged in a microfuge for 10 minutes. The supernatant was collected and the protein concentration was determined by DC protein assay (Bio-Rad). 30 μg protein, combined with 4% β-mercaptoethanol (β-ME) and 0.001% bromophenol blue, was loaded onto mini-gels after denaturation at 95°C for 3 minutes. Run at 35 mA per 15% SDS gel (Laemmli, 1970) for 1 hour, proteins were separated and transferred to a PVDF membrane (Schleicher-Schluel). Proteins were analyzed by Western blot with antibodies diluted in 5% milk in TBS/o.5% Tween-20 solution. Primary antibodies included polyclonal Po Ab at 1:3000 or undiluted myc-supernatant for overnight incubation at 4°C. Goat anti-rabbit/anti-mouse alkaline phosphatase-conjugated (1:15,000) (Sigma) or horse radish peroxidase (HRP)-conjugated (1:20,000) (Sigma) were used as secondary antibodies, accordingly, for an hour at room temperature. Membranes were developed by BCIP/NBT (KPL) substrate for the alkaline phosphatase assay or by enhanced chemiluminiscence (Amersham) for HRP.

**9. Deglycosylation of Po**

Cells at 80%-90% confluence on one 10-cm dish were lysed in 500 μl of 50 mM Tris-HCl (pH 8.6) containing 25 mM EDTA, 0.1% SDS, 0.5% NP-40 and 1% β-ME. The lysate was boiled for 3 minutes to denature proteins. Proteinase inhibitors mentioned previously were added when the sample was cooled down. Each sample was divided into half. To one half, 2 μl of peptide N-glycosidase F (PNGase F) (Boehringer-Mannheim Biochemical) was added, while the other half with no PNGase F added was as the control. Both were incubated at 37°C for at least 4 hours, then proteins were precipitated by 8 volumes of ice-cold acetone for 2 hours at -20°C. The protein pellet was collected by centrifugation at 3000 rpm for 20 minutes, air-dried, resuspended in 80 μl 1x SDS lysis buffer, and then subjected to SDS-PAGE. Same volumes of samples were loaded for each compared reaction. After the separation, the proteins were transferred to membrane, immunostained as described above.

**10. Detection of Po at the cell surface by indirect immunofluorescence**

Cells were grown on 8-chamber tissue culture slides (Lab-Tek) coated with poly-L-lysine and fibronectin. At 50% confluence, the cells were fixed with 4% paraformaldehyde for 30 minutes, rinsed 3x DMEM, then blocked in dDMEM for 30 minutes at room temperature. Primary Abs of rabbit anti-bovine Po polyclonal (1:50) or mouse anti-myc (Invitrogen) (1:200) diluted in dDMEM were added for an incubation at 4°C for overnight. After three washes with DMEM and one blocking, the cells were

incubated with phycoprobe (PE)-conjugated goat anti-rabbit Ig in a 1:50 (Biomeda) or fluorochrome FITC-conjugated goat anti-mouse Ig in a 1:100 (Santa Cruz) for an hour at room temperature. The slides were washed three times more in PBS, then mounted in Permfluor (Baxter) and viewed with a Zeiss fluorescent microscope or a Nikon confocal scanning microscope.

**11. Biotinylation of cell surface proteins**

Cells (80%-90% confluence) in 10-cm dish were washed with PBS three times and then incubated with 3 ml of 0.5 mg/ml sulfo-N-hydroxysulfosuccinimide(NHS)-biotin (Piece Chemical) dissolved in PBS for 30 minutes at room temperature. The labelling solution was removed and the cells were labelled one more time with fresh solution. Then the cells were washed three times with PBS, scraped-off the plate, and subjected to immunoprecipitation.

**12. Immunoprecipitation of Po**

Cells in a 10-cm dish were lysed in 500 μl RIPA buffer containing 50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.2% SDS, 1% deoxycholate and a cocktail of proteinase inhibitors. The nuclei were removed by centrifugation at 14,000 rpm for 10 min, and the supernatant lysates were measured for protein concentration. 500 μg of total proteins were taken and diluted to 1 ml using RIPA buffer. The lysates were pre-cleared with normal rabbit/mouse serum and Bugs (Pansorbin cells) (Calbiochem) at 4°C for 1.5

hour on a shaker. The Bugs were spun down and the lysates were incubated with 8 μl of Po polyclonal Ab at 4°C, overnight with gentle rocking. 50 μl of 10% Protein A sepharose (Pharmacia) was added to the mixture for another 2-hour incubation. For co-transfectants, prior to the application of Po Ab, the lysates were first incubated with 30 μl myc-beads (see method 1) for overnight at 4°C. The supernants, after separation from the beads by centrifugation at 14,000 rpm for 10 min, were then immunoprecipitated by Po-Ab. The beads were collected and washed three times with low-salt wash buffer containing 10 mM Tris, pH7.5, 2 mM EDTA and 0.5 M DTT. The samples bound on beads were deglycosylated with PNGase F, as described above, before being eluted from the beads. After extensive wash with PBS, the beads were resuspended in 30 μl of sample buffer and β-ME, boiled at 95°C for 3 min and spun again. The supernant was collected, electrophoresed on SDS-PAGE, transferred onto PVDF membrane and blotted with streptavidin-HRP. The enzyme reaction was visualized by the ECL method from KPL.

**13. Quantitation of Po expressed at the cell surface**

Cells with a population of 2,000-3,000/well were plated down in a 96-well plate and allowed to attach and grow for two days. ELISA (enzyme-linked immunosorbent assay) was carried out as the following: The cells were first fixed with 4% paraformaldehyde for 30 min, rinsed 3x with PBS, blocked with 3% normal goat serum (NGS) in dDMEM for 30 min, and then incubated with rabbit anti-bovine Po serum (1:200) in dDMEM containing 1% NGS at 4°C for overnight. The cells were rinsed and

blocked again, and then incubated with HRP-conjugated goat anti-rabbit Ig (1:1000) (Sigma) for one hour at room temperature. The cells were rinsed with abundant PBS. Colour was developed by the addition of 50 μl of 0.2% (wt/vol) o-phenylenediamine (OPD) (Sigma) and 0.2% (wt/vol) $H_2O_2$ in citate buffer (pH 5.0). The reaction was stopped after 15 minutes by the addition of 4.5 M $H_2SO_4$, and the optical density at 490 nm was read immediately by a 96-well microplate reader (Bio-Tek Instrument). For each plate, one row of 12 wells was devoid of addition of either primary or secondary antibodies, their readings, taken as the blank, were subtracted from the other readings on the same plate. Results were standardized to absorbance units per cell. The average number of cells per well was estimated by counting cells using a Coulter counter after removing them with trypsin, from 5 separate wells for each 96-well plate. For each cell line, the results were considered consistent after at least three independent experiments.

**14. Adhesion assay**

Cells at 80%-90% confluence were washed with PBS and incubated with 5U/ml trypsin (GIBCO) in PBS for 2-3 minutes at room temperature to be detached from the plate. The cells were resuspended in DMEM, spun at 3000 rpm for 5 min, and then washed twice with DMEM. Finally the cells were resuspended in 2 ml of dDMEM by 4 passages through an 18-gauge syringe. After the cells were counted, the suspensions, containing 95% single cells, were diluted to a final concentration of $1.5\text{-}2.0\text{x}10^6$ per ml and allowed to aggregate at 37°C with gentle rocking at 10 rpm. Before sampling, the tubes were gently inverted and aliquots were removed at intervals, examined under the

microscope, and the total particle number was determined with a Coulter counter. At least three separate incubation were performed for each experiment, and duplicate samples were withdrawn at each time point and counted three times each.

# Chapter III

# Characterization of Mutated Human Po Proteins

## 1. Introduction

The CMT1B locus was mapped to chromosome 1q21-23 in the early 1980s (Bird *et al.*, 1982). It was only when the human Po gene (*MPZ*) was assigned to the same region (Hayasaka *et al.*, 1993) that linkage of Po with CMT1B was pursued (Hayasaka *et al.*, 1993; Su *et al.*, 1993; Kulkens *et al.*, 1993). Po, the most abundant protein in PNS myelin, has been suggested to be responsible for membrane adhesion and compaction of PNS myelin (Ganser and Kirschner, 1980; Kirschner and Ganser, 1980). Evidence *in vitro* (D'Urso *et al.*, 1990; Filbin *et al.*, 1990; Schneider-schaulies *et al.*, 1990) and *in vivo* (Giese *et al.*, 1992) support this suggestion. The association of the human demyelinating disease with the defects in the Po gene further confirms the importance of Po in compaction and physiological function of PNS myelin.

The human *MPZ* gene spans about 7 kb and contains six exons, each corresponding to a proposed functional domain; exon 1 corresponds to the signal sequence, exon 2 and 3 to the extracellular domain, exon 4 to the transmembrane domain and exon5 and 6 to the cytoplasmic domain. To date, all exons, except exon 1, are reported to carry Po mutations in CMT patients. The Ig-like extracellular domain, encoded by exon 2 and 3, contains the most mutations, of which mostly are missense mutations. These mutations are distributed throughout the whole extracellular domain, with the exception of β-strands A', B, C'' and inter-strand loop C'C''. Since Po behaves as a homophilic adhesion molecule via the interaction of its extracellular domain (Filbin *et al.*, 1990), mutations at the extracellular domain probably adversely affect Po's adhesive function. As the mutations are not clustered in any one region, it is likely that

some mutations affects the adhesive binding site directly but others may disrupt adhesion by affecting the conformation of the molecule.

As most CMT patients are heterozygotes for the mutated Po gene and different degrees of severity are associated with different pedigrees, there are at least three possible mechanisms whereby the half dose of mutated Po protein can affect myelin. First, the mutated protein does not reach myelin but is held up in the Schwann cell body. Consequently, only half the normal amount of Po would reach myelin and the disease is likely to be a result of half a gene dose. In addition, accumulation of the mutated Po in the Schwann cell could have a detrimental effect. Second, the mutated Po is not adhesive, reaches myelin, but has no effect on the functioning of the wild type Po protein. Again, the phenotype would be a consequence of reduced levels of Po gene dose. Third, the mutated Po protein is not adhesive, reaches myelin but has a dominant-negative effect on the functioning of the wild type protein. It is predicted that the simple dose effect only brings about the milder phenotype while the dominant-negative effect will produce the more severe phenotype. Indeed, the $Po^{+/-}$ heterozygotic mouse, lacking one copy of the normal Po gene, shows signs of demyelination at 4 months of age, which mimics a late-onset, mild form of the disease (Martini *et al.*, 1995).

How individual mutations affect the adhesive function of Po protein and bring about the disease phenotype can be tested *in vitro* using the CHO cell transfection/aggregation system. In our laboratory, transfected CHO cells have been used to characterize the behaviour of Po mutants. Initially, the role of the single carbohydrate chain of Po in adhesion was addressed (Filbin and Tennekoon, 1991; 1993). Subsequently, the integrity of the cytoplasmic domain (Wong and Filbin, 1994; 1996)

and then the formation of the disulfide bond of the Ig-domain were studied (Zhang and Filbin, 1994; 1996). Furthermore, a sequence directly involved in adhesion within the extracellular domain of Po was also identified (Zhang *et al.*, 1996). It has been proven that this aggregation/adhesion system is ideal for carrying out such studies. Therefore, a similar strategy will be applied to the study of CMT1B mutations. Six mutations corresponding to six CMT1B pedigrees were selected. Two mutations, Ser34 to Cys (Hayasaka *et al.*, 1993b) and deletion of Ser34 (Kulkens *et al.*, 1993), occur at the same amino acid. Interestingly, they gave distinct phenotypes in CMT patients. Substitution of Ser34 with Cys produces the more severe DSS while deletion of Ser34 presents the milder CMT1B. So Ser34 provides an excellent model to examine how disease severity may correlate with a particular mutation and how that mutation affects the function of Po. Another two mutations, Asp61 to Glu and Lys67 to Glu, are chosen because they are among the few mutations first identified in CMT patients (Hayasaka *et al.*, 1993a) when we started the project. Furthermore, although one is a conservative substitution and the other has a dramatic change of charge, both gave a similar CMT1B phenotype (Hayasaka *et al.*, 1993a). The other two mutations lead to the replacement of Tyr125 (Y125) or Tyr152 (Y152) with a stop codon (Nelis *et al.*, 1994). Both mutations are associated with the mild CMT phenotypes. Tyr152 is located at the junction of the transmembrane domain with the cytoplasmic domain, so the non-sense mutation will truncate the entire intracellular domain of Po protein. In contrast, Tyr125 is located at the junction of the extracellular domain with the transmembrane domain, therefore the non-sense mutation will produce a protein with only the extracellular domain. It will be interesting to determine if the resulting truncated proteins reach the cell surface and if the associated

CMT phenotypes are due to a loss-of-function. To gain insight into these mutations, the mutated Po cDNA will be transfected into CHO cells and using the adhesion assay, the effect of these mutations on Po adhesion will be assessed.

## 2. Results

### 2.1. Expression of wild type human Po

Po has been shown to behave as a homophilic adhesion molecule via interactions of its extracellular domain (Filbin *et al.*, 1990). This was carried out by expressing rat Po cDNA in non-adherent CHO cells and showing they aggregated much more readily than the control cells. Human Po protein shares more than 94% sequence similarity with the

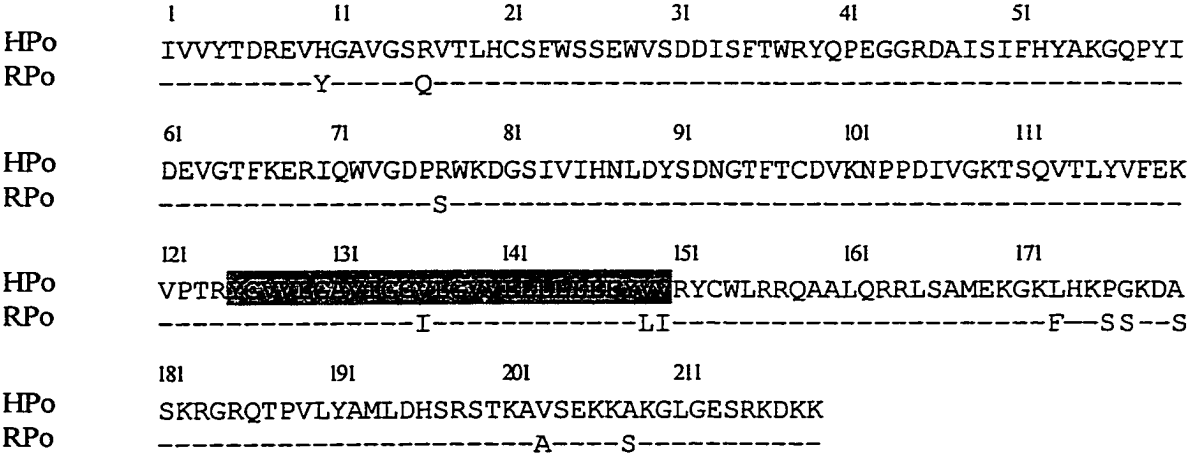



Fig.5: Comparison and alignment of Po amino acid sequence from human (Hayasaka *et al.*, 1993) and rat (Lemke *et al.*, 1988). Numbers are given to matured protein sequences. Transmembrane domain is shadowed and nonidentical amino acids are indicated.

rat Po (Fig.5). It is predicted that human Po will function in the same way. However, direct *in vitro* adhesion data is lacking. Therefore, a 1.12 kb sequence containing the entire human Po cDNA (hPocDNA) was cloned into the vector pSJL at the XhoI site, either in a 5'→3' or a 3'→5' orientation and the hPocDNA-containing vector was introduced into CHO cells by transfection. Transfectants were selected in G418 and then single-cell cloned. Expression of Po by a dozen of clones was amplified with MTX and the expression level was monitored by Western-blot of the cell lysates. Figure 6 shows the results from the selected clones. One clone (Fig.6, lane 5), which expressed a comparable amount of protein to the clone expressing the rat Po (RPo) (Fig.6, lane 1), which we previously had shown to be adhesive, was chosen for further study. The adhesive properties were quantitated by counting the total particle number of a cell suspension as incubation proceeds; a drop in total particle number indicates aggregation formation. Figure 7 shows the total particle number decreases to 25% in 60 min for the hPo-expressing cells, to 45% for the rPo-expressing cells, but to only 80% for the control cells over the same time. These results suggest that the cells expressing the human Po protein are as adhesive as the cells expressing the rat Po.

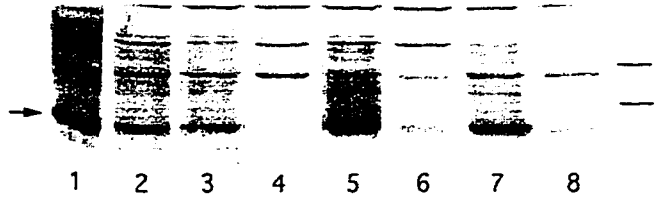



Fig.6: Western blot screening for clone expressing wild type human Po. Lane 1, control cells, cells expressing wild type rat Po; Lane 2-8, cells trnasfeted with wild type human Po cDNA. For each lane, 30 μg of total lysate protein was used. Arrow refers to Po. Bars indicate molecular weight standards from top to bottom as follows: 38, 32 KD.

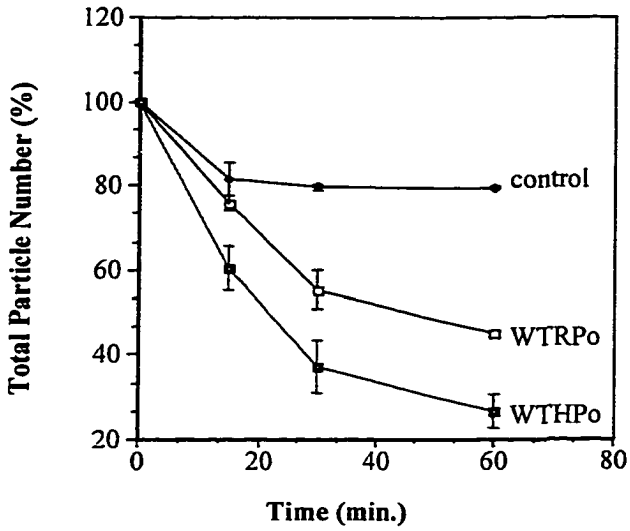



Fig.7: Aggregation properties of Po-expressing cells. Single-cell suspensions of CHO cells expressing wild type rat Po, control-transfected cells, or wild type human Po were allowed to aggregate. Samples were withdrawn at intervals to determine the total particle number as counted by a Coulter counter. The total particle number ± S.E. was plotted against time.

## 2.2. Expression of mutated HPoS34C

To test whether the mutations found in CMT patients affect the adhesion of Po, a series of site-directed mutagenesis of specific amino acids was performed. The mutation work began with Ser34, an amino acid whose functional importance is suggested by the observation that three distinct mutations in the CMT are associated with this site (Hayasaka *et al.*, 1993; Kulkens *et al.*, 1993; Blanquet-Grossard *et al.*, 1995). Substitution of Ser34 with Cys was carried out via a two-step PCR, and the mutated cDNA fragment was subcloned into the pSJL vector and transfected into CHO cells. After gene selection by G418 and amplification by the dhfr/MTX strategy, single-cell cloning was carried out. Figure 8 shows expression of S34-mutated human Po proteins, termed hPoS34C, by eight single cell clones on Western-blot.

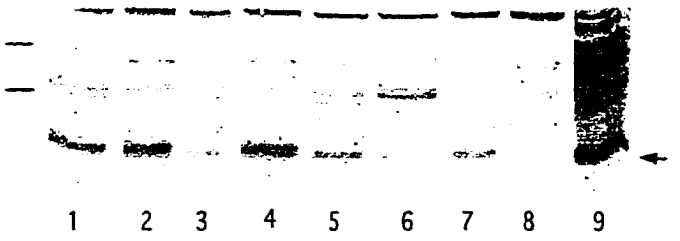



Fig.8: Western analysis screening for cells expressing HPoS34C. Lane 1-8, cells transfected with HPoS34C cDNA; Lane 9, control cells expressing wild type rat Po. For each lane, 30 μg of total lysate protein was used. Arrow refers to Po. Bars indicate molecular weight standards from top to bottom as follows: 38, 32 KD.

## 2.3. Glycosylation and surface expression of wild type hPo and hPoS34C

One clone of hPoS34C-expressing cells (Fig.8, lane 1), which expressed approximately the same amount of protein as the wild type hPo-expressing clone (Fig.6, lane 3), was chosen for further studies.

It was previously shown that rat Po in transfected cells was glycosylated and the sugar was required for the adhesion of cells to take place (Filbin and Tennekoon, 1991). To examine whether the wild type hPo and hPoS34C were each glycosylated, the cell lysates were treated with PNGase F, an enzyme which cleaves all N-linked sugars between the innermost GlcNAc and asparagine residue. Figure 9 shows that after PNGase F treatment, the molecular weight of hPoS34C decreased by the same extent as the wild type hPo after the removal of the sugar, i.e. by approximately 6%. This indicates that both wild type hPo and hPoS34C are glycosylated and that the extent of glycosylation is similar in each.

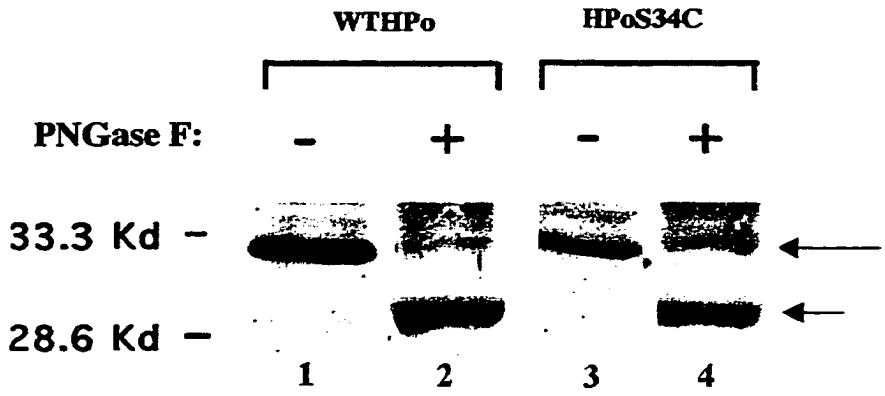



Fig.9: Effect of treatment of transfected CHO cell lysates with PNGase F. Proteins (30 μg/lane) from cell lysates, with (+) and without (–) treatment with PNGase F, transfected with wild type human Po cDNA (lane 1 and 2), or with mutated HPoS34C (lane 3 and 4), were separated by SDS-PAGE and immunostained for Po. Long arrow refers to glycosylated Po, short arrow, deglycosylated Po.

As the homophilic cell-cell adhesion mediated by Po is dependent on the presence of Po at the cell surface, the adhesion of hPo and hPoS34C could be compared only after they were shown to express equivalent levels of Po protein at the cell surface. To confirm that Po was reaching the cell surface in the two cell lines, immunofluorescence of intact cells was carried out. Figure 10 shows indirect immunofluorescence staining of fixed, unpermeabilized transfected cells with Po antibody, that the intensity of staining was similar for cells expressing hPo and hPoS34C. The surface expression of the two proteins was then quantitated. An ELISA was carried out on fixed, unpermeabilized cells. The hPoS34C expressing cells were shown to express comparable amounts of Po on the cell surface as the cells expressing the wild type hPo, while the control cells, not

expressing Po protein, gave only a background signal (Fig.11). Therefore, these results suggest that these two cell lines are appropriate for comparing their adhesive properties.

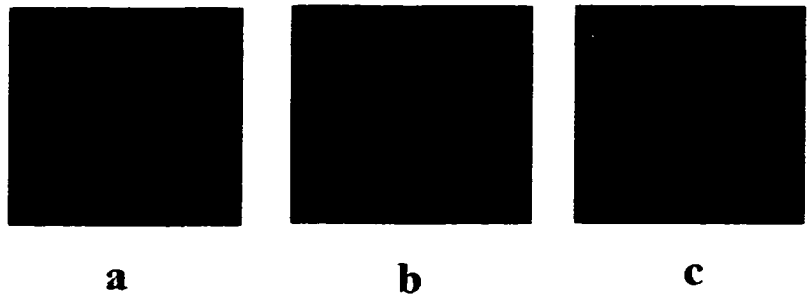



Fig.10: Surface detection of Po on intact cells by indirect immunofluorescene: (a) cells expressing wild type human Po; (b) cells expressing mutated HPoS34C. and (c) control transfected cells.

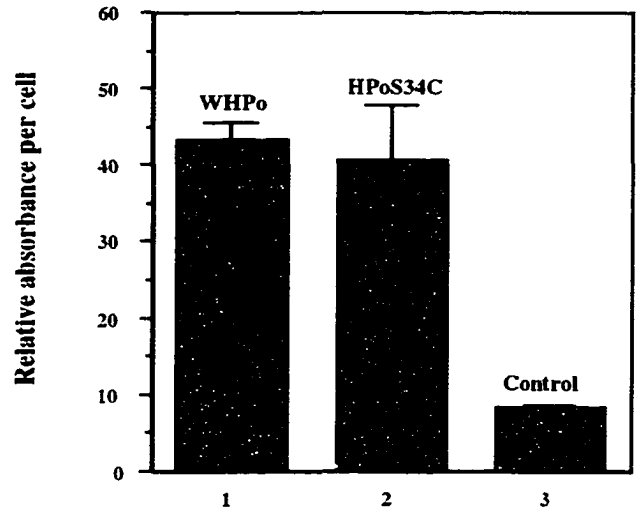
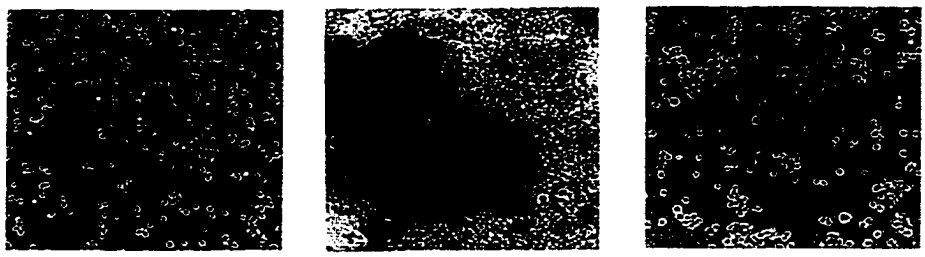



Fig.11: Quantitation of Po expressed at the cell surface. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected cells expressing wild type human Po (column 1, WHPo), mutated Po (column 2, HPoS34C) and control-trnasfected cells (column 3, control), using a Po-peptide antibody directed against sequences in the extracellular domain. Results are expressed (±S.E.) in relative absorbance units per cell and are the mean of three experiments, 32 samples per experiments.

## 2.4. Comparison of adhesive properties of hPoS34C expressing cells with the wild type hPo-expressing cells

Once the cell lines expressing equivalent amounts of hPoS34C or hPo on the cell surface were established, their adhesive properties were compared. This was carried out by the same aggregation/adhesion assay as we mentioned previously. The cells expressing wild type hPo, mutated hPoS34C, or control cells were obtained in a single-cell suspension and aggregation was allowed to proceed while gently rocking at 37°C. After various times of incubation, aggregate formation was checked under the microscope and the total particle number was counted with a Coulter counter. A drop in total particle number indicates aggregate formation.

By 90 minute of incubation, the total particle number for the cells expressing wild type hPo dropped to approximately 20% of the starting cell number (Fig.12) and the cells formed large aggregates. In contrast, the cells expressing mutated hPoS34C, in the same time did not form large aggregates but remained as single cells, doublets or triplets and the total particle number dropped to only 65% of the starting value, which was indistinguishable from the control cells not expressing Po protein. These results indicate that mutation of Ser34 to Cys abolishes the adhesive properties of human Po protein.



a b c

d.

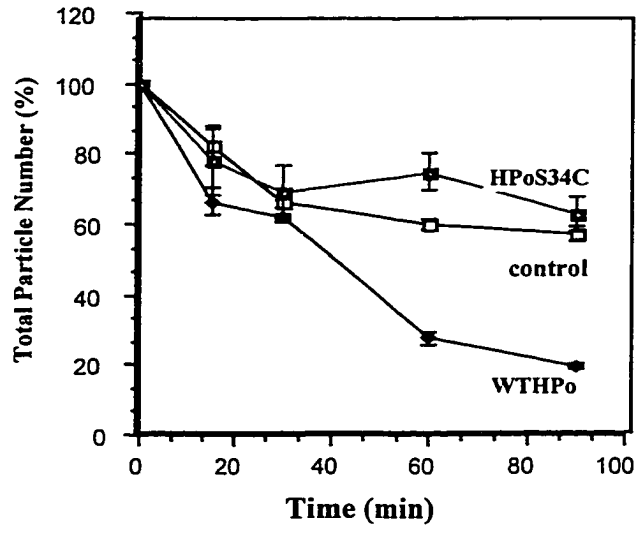



Fig.12: Aggregation properties of Po-expressing cells. A single cell suspension of cells expressing (a) control transfected cells, (b) wild type HPo, or (c) mutated HPoS34C was allowed to aggregate. Samples were withdrawn at various intervals and examined under microscope (a-c represent results after 60 min aggregation) and counted in a Coulter counter for total particle number. The percentage drop in total particle number ± S.E. was plotted against time (d).

## 2.5. Characterization of hPoD61E and hPoK67E

To determine if the residues Asp61 or Lys67 are each essential for Po protein to adhere, Asp61 and Lys67 were each replaced by Glu. These are the same substitutions found in CMT patients. The cDNA for Asp61-mutated Po (hPoD61E) or Lys67-mutated Po (hPoK67E) was each transfected into CHO cells. Individual clones were selected, amplified and screened as described above for hPoS34C. Figure 13 shows expression of

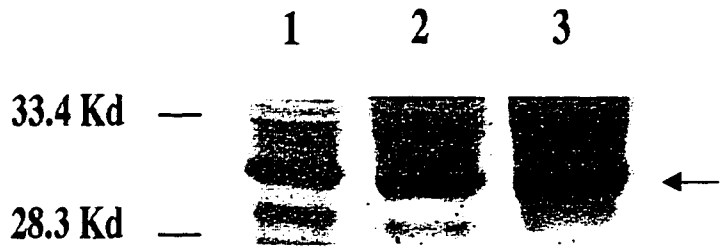



Fig.13: Western blot immunostained for Po of cells expressing wild type HPo protein (lane 3), mutated HPoD61E (lane 2) and HPoK67E (lane 1). For each lane 30 μg of total proteins was used. Arrow refers to Po.

the mutated Po protein by one such clone from each transfection. Both the hPoD61E-expressing clone (lane 2) and the hPoK67E-expressing clone (lane 1) showed approximately the same level of Po expression as the clone expressing the wild type Po (lane 3). These clones were then examined and quantitated for surface Po expression by an ELISA of fixed, unpermeablized cells (Fig.14). It was shown that transfected CHO

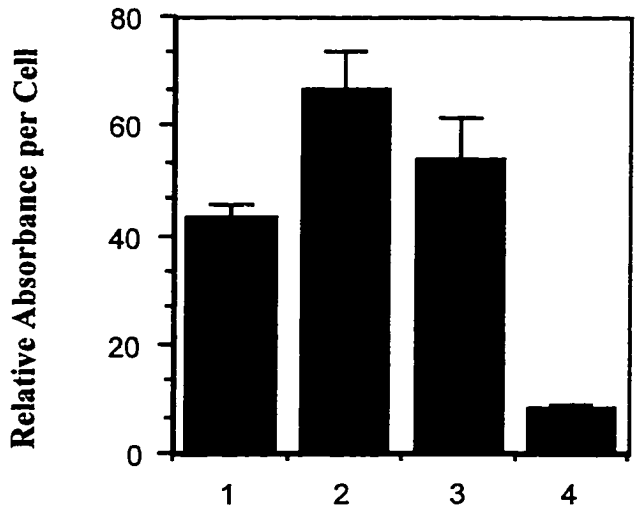



Figure 14: Quantitation by ELISA of Po expressed at the cell surface of transfected CHO cells. An ELISA was carried out on fixed, unpermeabilized cells. Column 1, cells expressing wild type HPo; 2, cells expressing mutated HPoK67E; 3, cells expressing mutated HPoD61E; 4, control transfected cells. The mutated Po proteins are reaching the cells surface in amount more than the normal Po protein.

cells expressing either hPoD61E or hPoK67E expressed about 25% to 55 % more Po at the cell surface (Fig.14, column 3 and 2, respectively), than the cells expressing the wild type hPo (Fig.14, column 1). Only background staining was apparent in the control-transfected cells when the same antibody was used (Fig.14, column 4). It has been shown that the level of expression of wild type hPo by the cell line represented in Fig.14, column 1, is sufficient to measure the homophilic adhesion of this molecule (Fig.12). Therefore, because the cell lines expressing the mutated Po cDNAs each expressed even more Po than the cells expressing the wild type Po, they are suitable for assessment of adhesion. The adhesive properties of the cells expressing these mutated Po's were compared to those of the cells expressing the wild type Po. After 60 minutes incubation of a single-cell suspension, the total particle number of the cells expressing the wild type hPo dropped by about 65% of the starting number. In contrast, the total particle number

of the cells expressing either hPoD61E or hPoK67E dropped by only about 25% in the same time. These results are presented as a percentage of specific adhesion after a 60-min incubation, with 100% adhesion taken as adhesion of the cells that expressed the wild type hPo (Fig.15). It was shown that the specific binding for the control cells was about 60% while for hPoD61E and hPoK67E that was only about 40%. Therefore, these results suggest that mutation of Asp61 or Lys67 abolishes or greatly reduces the adhesive properties of the wild type Po.

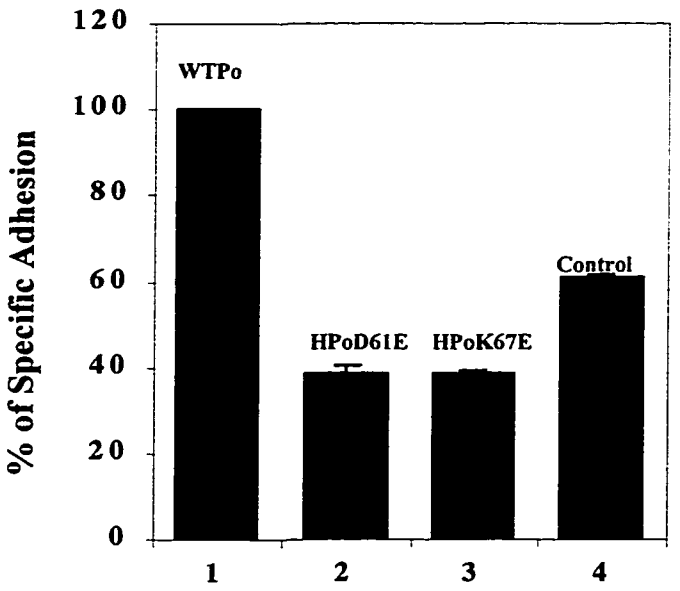



Fig.15: Aggregation properties of Po-expressing cells. Aggregation was allowed to proceed as described in Figure 3 and 8 except that the samples were withdrawn only at start of incubation and after a 60 min incubation period, and the total particle number was countered in a Coulter counter as $N_0$ and $N_{60}$ accordingly. The percentage drop in total particle number of the cells was related to $N_{60}$-$N_0/N_0$. The specific adhesion was calculated for HPoD61E (column 2) and HPoK67E (column 3)-expressing cells and the control cells (column 4) as the percentage drop in total particle number of the cells expressing the wild type HPo (column 1) was taken as one hundred percent specific binding. The results represent the mean of at least three experiments.

## 2.6. Characterization of hPoY125stop and hPoY152Stop

The non-sense mutation of Tyr125 or Tyr152 of Po was carried out on hPo-cDNA by site-directed mutagenesis, respectively. The mutated Po cDNAs in vector pSJL were transfected into CHO cells and G418-resistent clones were selected.

The cDNA coding for the mutated Po protein, hPoY125stop (hPoY125X) or hPoY152stop (hPoY152X), was amplified by gradually increasing the concentration of MTX to 1.0μM, a concentration at which all other mutated Po proteins have been strongly expressed. Cell lysates from hPoY125X or hPoY152X cDNA-transfected cells were screened for Po expression on a Western-blot. The molecular weight for unglycosylated hPoY125X and hPoY152X was predicted to be around 14.2 kd and 17.0 kd, respectively. To obtain better resolution for these small molecules, a tricine SDS-PAGE gel system (Schägger and von Jagow, 1987) was used instead of the usual glycine SDS-PAGE. On the Western, no bands were detected in the range of 10-24 kd by Po-antibody for six randomly chosen clones from each transfection (data not shown). To assess if mutated proteins were secreted into the media as soluble forms, the media were collected from one such clone for both hPoY125X and hPoY152X, and incubated with Po-antibody and protein A-sepharose beads for overnight at 4°C. The beads were spun down and the elutes were loaded onto the gel and viewed by silver-staining. It was found that when compared to the negative control, the cells not expressing Po, no additional proteins were precipitated with the Po-antibody from the hPoY125X and hPoY152X cell media (data not shown).

Non-detection of Po protein could be due to no/low expression of Po mRNA. Therefore, these clones were then examined for transcription of Po mRNA. This was carried out by RT-PCR. Total RNA was isolated from hPoY125X, hPoY152X and the control rPo-expressing cells. After reverse-transcription, a coding region of Po was amplified with a pair of primers corresponding to the hPo cDNA sequence. The PCR products were subjected to agarose gel electrophoresis. Figure 16 shows that a fragment of expected size (490 bp) was obtained for both hPoY125X and hPoY152X clones (lane 2 and 3, respectively). In contrast, the rat Po cDNA was not amplified (lane 1). This was because the primers were designed specifically for human Po cDNA and not for rat. However, since there are only three-nucleotide difference on the corresponding sequence between human and rat Po cDNAs, if the PCR condition was adjusted to less stringency, the rat Po cDNA could be amplified too (Fig. 16, lane 7). Further analysis of the PCR products was carried out with *RsaI* digestion, as each mutation destroys a *RsaI* recognition site within the amplified fragment. It is predicted that for the non-mutated sequence, the *RsaI* treatment would generate three fragments: 283 bp, 128 bp and 80 bp. However, as a result of the mutation, two fragments will be produced instead of three. Figure 16, lane 5 and 6 shows the results. As the two *RsaI* sites are separated by 80 bp, for the non-sense mutation of Y125, two fragments of 363 bp and 128 bp were generated (lane 5), while for the nonsense mutation of Y152, two fragments of 283 bp and 208 bp were generated. These results not only indicate the presence of the mRNAs for the hPoY125X and hPoY152X cDNAs, but also confirm the presence of the mutation at the correct position. Although this RT-PCR is not the quantitative one, the presence of Po mRNA should reflect its abundance, as the site-directly-mutated Po mRNA is unlikely to

be degraded. An adhesion assay was performed with these cells even though no mutated Po was detected. No adhesion was demonstrated for either of these two cell lines (data not shown).

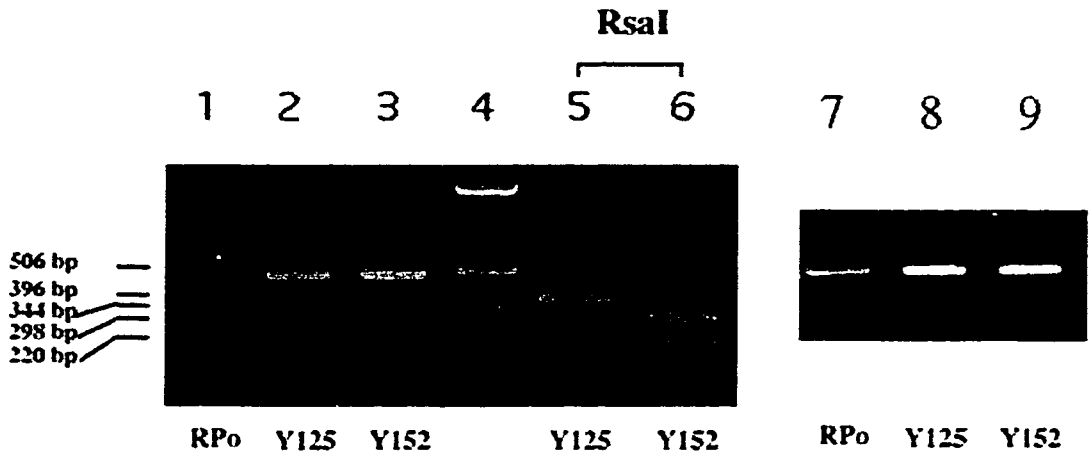



Fig.16: Detection of HPo mRNA using RT-PCR. The total RNA was isolated from the transfected cells and reverse-transcribed. Po cDNA was amplified using Po-specific primers. RT-PCR products were analyzed by electrophoresis. Lane 1 and 7, cells expressing rat Po; lane 2 and 8, cells transfected with HPoY125stop cDNA; lane 3 and 9, cells transfected with HPoY152stop cDNA. Lanes 1, 2 and 3 are PCR products performed under stringent conditions while lanes 7, 8, and 9 are under less stringent conditions. Lane 5 and 6, RsaI digestion of PCR products from lane 2 and 3, respectively. Lane 4, molecular weight marker with numbers on the side.

## 2.7. Non-adhesiveness of hPoΔS34-expressing cells

It was stated previously that in affected patients, substitution of Po Ser34 with Cys or deletion of Ser34 is associated with a severe and a mild form of CMT1B, respectively. In order to compare the behaviour of these two mutated Po in transfected

cells, deletion of S34 was also carried out. Figure 17A shows expression of the S34-deleted hPo (hPoΔS34) by one single-cell clone after growth in increasing concentration of MTX to a final concentration of 0.2 μM. As hPoΔS34 on the Western-blot appeared to have the same molecular weight as the wild type Po, it is likely that hPoΔS34 is glycosylated to the same extent as the wild type Po. It has been noticed that it took unusually long for this cell line to be established as the cells survived poorly at each increased concentration of MTX, especially when concentration of MTX was increased directly from 0.2 μM to 0.5 μM without a more gradual increase. It is known that a high concentration of MTX is toxic to the cells that do not have a high copy number of the *dhfr* gene. However, this long has never been experienced. There is a possibility that some cells with multiple copies of the *dhfr* gene, and accompanying hPoΔ34 cDNA, died of possible toxic effects because of accumulation of hPoΔS34. At a concentration of 0.2 μM MTX, the hPoΔS34-expressing cells have shown to express as much Po as the cells expressing the wild type Po. Surface staining by Po-antibody also confirmed that the Po surface expression levels between the hPoSΔ34-expressing cells and wild type Po-expressing cells are comparable (Fig.17B). Therefore, an adhesion assay was performed with these cells. Figure 17C shows that after 60-min incubation, the total particle number for the cells expressing the wild type Po dropped to about 15%. In contrast, the total particle numbers for hPoΔS34-expressing cells or the control cells dropped to about only 55% in the same time. This result implies that deletion of S34 abolishes the adhesive properties of wild type Po.

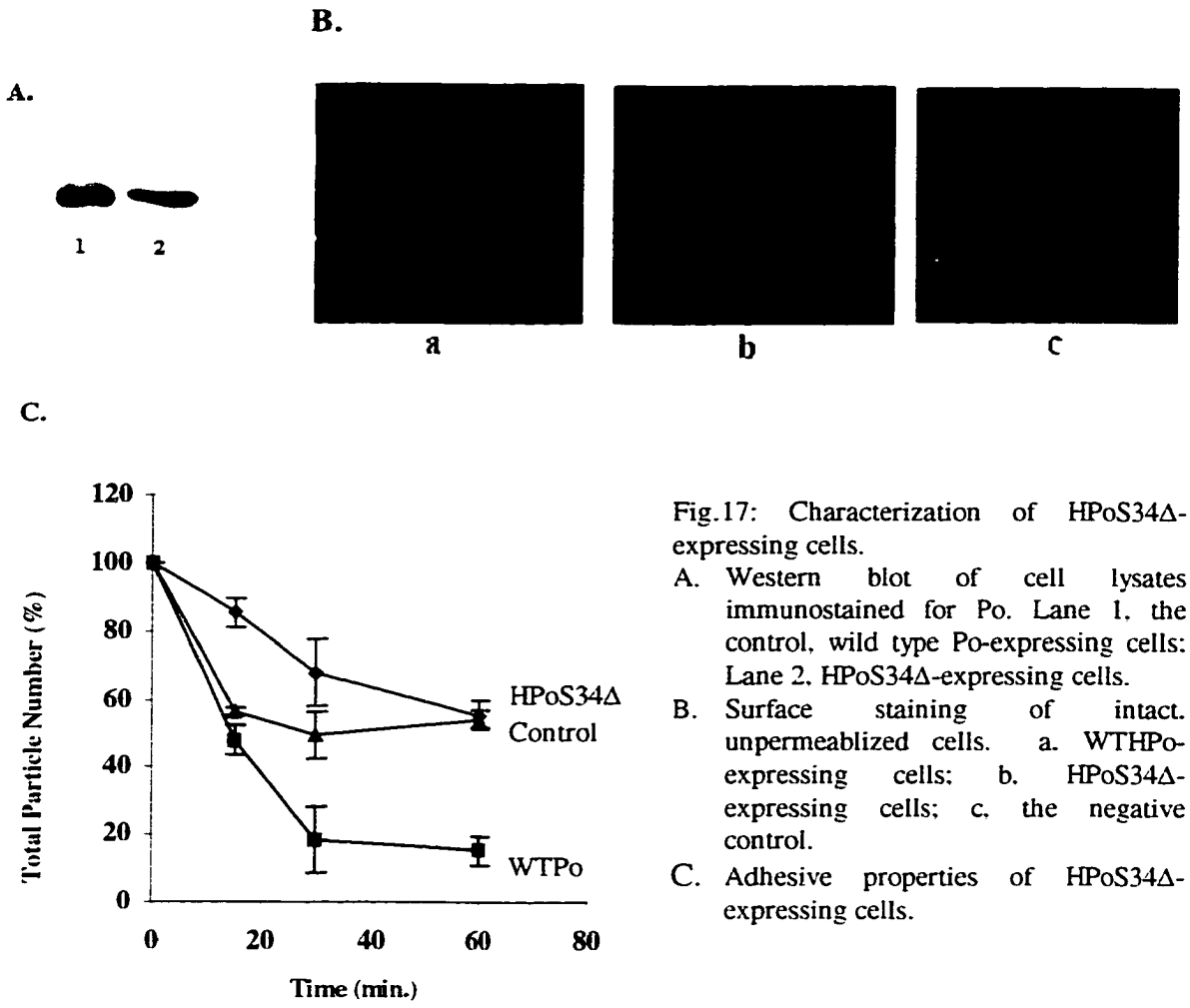



Fig.17: Characterization of HPoS34Δ-expressing cells.

A. Western blot of cell lysates immunostained for Po. Lane 1, the control, wild type Po-expressing cells: Lane 2, HPoS34Δ-expressing cells.

B. Surface staining of intact, unpermeablized cells. a. WTHPo-expressing cells; b. HPoS34Δ-expressing cells; c. the negative control.

C. Adhesive properties of HPoS34Δ-expressing cells.

## 3. Summary

1) Wild type human Po protein expressed in CHO cells shows strong adhesive properties.
2) Six human Po mutations were constructed corresponding to mutations in human Po in CMT1B. Po Ser34 was replaced by Cys or was deleted, Asp61 by Glu and Lys67 by Glu. Both Ty125 and Tyr152 were substituted with stop codons.
3) HPoS34C, D61E, K67E and S34Δ proteins were expressed by CHO cells on the cell surface, at levels comparable to the surface expression of the wild type Po.
4) HPoY125X and Y152X proteins were not detected either in the cell membrane nor secreted into the media. However, their mRNAs were detected.
5) HPoS34C was shown to be glycosylated to the same extent as the wild type hPo protein.
6) Cells expressing hPoS34C, D61E, K67E and hPoΔS34 did not adhere homophilically.

# Chapter IV

# Characterization of Dominant-Negative Effects of Mutated Po Proteins

## 1. Introduction

In chapter III we showed that Po with point mutations at Ser34, Asp61 or Lys67, or a deletion at Ser34, or nonsense mutations at Tyr125, Tyr152 had no adhesive properties. However, for CMT1B patients with these mutations, the mutations only affect one allele of the chromosome. Hence, only 50% of the Po protein is aberrant while the other 50% is normal. Three possible mechanisms whereby heterozygote mutations in Po could affect myelin and cause the disease have been proposed. In the first mechanism, the simple reduced dose effect could apply to the non-sense mutations of Tyr125 and Tyr152, as the mutated Po proteins might not reach the membrane. As a consequence, only half the amount of Po for normal individuals is present on the cell surface. It has been demonstrated in the heterozygous $Po^{+/-}$ mice that only one copy of Po gene is not sufficient to maintain PNS myelin (Martini *et al.*., 1995). Therefore, not enough wild type Po protein could be the reason for the disease. However, for patients carrying the mutations such as S34C, D61E and K67E another mechanism must apply, as the aberrant proteins appear to reach the cell membrane and may exert a dominant-negative effect on the normal Po. Furthermore, different degrees of dominant-negative effect may be expected since patients with these mutations exhibited different severity of the disease. For example, mutation of S34C resulted in the severe DSS while mutations of the other two amino acids resulted in the milder CMT. How these mutated Po proteins affect the function of normal Po can be addressed by co-expressing the wild type Po with the mutated Po in the same cell and then examining the adhesive properties of co-expressors in compared to cells expressing only the wild type Po. Co-transfection will be carried out

using equal amounts of wild type Po cDNA and mutated Po cDNA. Since the mutated Po proteins have the same molecular weight as the wild type Po, it is impossible to distinguish one from the other based on molecular weight or available Po antibodies. Therefore, wild type rat Po with a myc-tag at the N-terminus will be used for cotransfection purposes. In addition to the above mutations, one more mutation will be added to the co-transfection studies. It was previously shown that mutation of Asn93 to Ala (N93A) abolished Po's adhesive properties (Filbin and Tennekoon, 1993). Although N93A is not yet found in any CMT patients, mutation of Asn93 to Ser (N93S) was reported in one pedigree of CMT1B patients (Blanquet-Grossard *et al.*., 1996). As both substitutions occur at the same position and replacing amino acids are both hydrophobic and similar in size, N93A mutation could represent the mutation found in CMT and be used for investigation of dominant-negative effects.

## 2. Results

### 2.1. Myc-tagged wild type Po functions in the same way as the untagged, wild type Po

To co-express the wild type and mutated Po protein, one problem we will encounter is how to confirm both types of Po are expressed. There is only one amino acid difference between the two types of Po protein and on a Western-blot, mutated Po proteins run at the same position as the wild type Po. Therefore, epitope tagging of at

least one of the two will help to solve the problem. Derived from the human c-myc protein, one of a family of nuclear proteins that has been found in several types of human tumors (Alitalo *et al.*., 1983), the Myc-epitope has been widely used to tag proteins for expression experiments in nonhuman cells. This is because the monoclonal antibody against the c-myc epitope is well-characterized (Evan *et al.*., 1985). Originally developed to study c-myc, anti-c-myc specifically recognizes the Myc-epitope sequence |EQKLISEEDL| and does not cross-react with other cellular proteins. We obtained the Myc-tagged wild type Po (MycPo) cDNA from Dr. Lawrence Wrabetz, Italy. Rat Po cDNA was inserted with a 40-bp myc-sequence and when the Po protein is processed, the Myc-tag will be at the N-terminal.

To verify that tagging of the myc-sequence to Po will not interfere with the adhesive function of the wild type Po, MycPo cDNA was transfected into CHO cells in the same way as described previously. Cell lines were obtained using the dhfr/MTX strategy and subsequent single cell cloned. After probing with polyclonal anti-Po antibody on a Western-blot (Fig.18), it was shown that one such clone expressed a protein of 30-32 kd (lane 2, arrow), a position slightly higher than that for untagged Po (lane 1, arrowhead). The same gel was then re-probed with monoclonal anti-myc antibody. The same protein was also recognized by myc antibody (lane 4) but the untagged Po was not (lane 3). This indicates that this protein is myc-tagged Po and that its increased molecular weight is due to the additional 13-peptide myc tag.

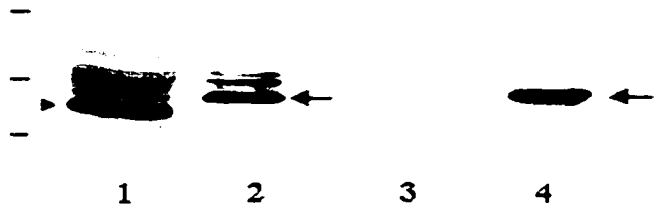



Fig.18: Immunodetection of MycPo protein after Western blotting of total cell lysate. Lane 1 and 3, cells expressing untagged, wild type RPo; lane 2 and 4, cells expressing myc-tagged, wild type RPo. Lane 1 and 2 are stained with Po-antibody and anti-rabbit AP; lane 3 and 4 stained with anti-myc antibody and anti-mouse HRP. Arrowhead refers to untagged, wild type Po, arrow refers to MycPo. Bars indicate molecular weight standards of 48, 33, and 28 KD.

Surface expression of MycPo was assessed by indirect fluorescence whereby unpermeablized, fixed cells were incubated with myc antibody followed by a fluorescent dye-conjugated second antibody (Fig.19). The bright staining indicates that the MycPo protein has reached the surface of these cells.

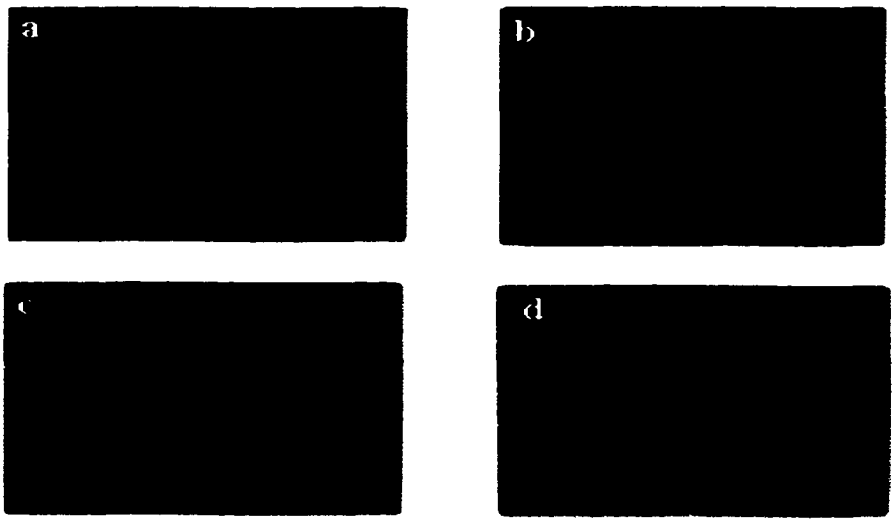
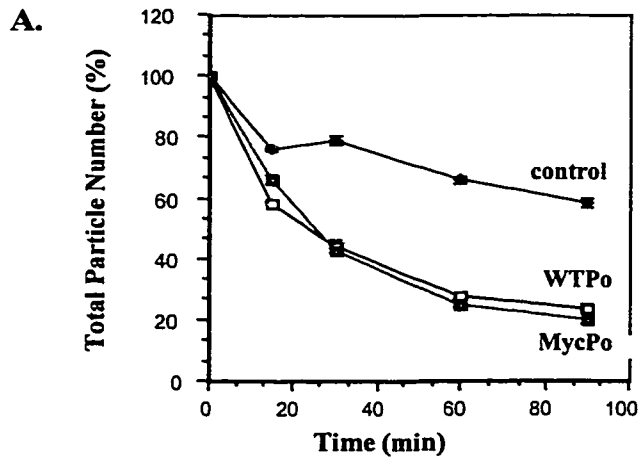



Fig.19: Detection of mycPo on intact cell surface by indirect immunofluorescene. (a,b) cells expressing myc-tagged. wild type RPo stained with myc-Ab (a) and Po-Ab (b); (c,d) cells expressing untagged, wild type RPo stained with myc-Ab (c) and Po-Ab (d).

Since the MycPo-expressing clone showed the same strong surface expression of Po as the untagged wild type Po-expressing cells (Fig.19), an adhesion assay was carried out as described above. By 90 minutes of incubation, the total particle number of the cells expressing MycPo had dropped to 20% of the starting particle number and formed large aggregates. This behaviour was indistinguishable from that of the control, untagged wild type Po (Fig.20A). Furthermore, in a mixed aggregation assay whereby a single-cell suspension of the lipophilic dye DiI (red)-labelled MycPo-expressing cells were mixed with a single-cell suspension of DiO (green)-labelled, untagged, wild type Po-expressing cells and incubated together, they formed large aggregates composed of both types of cells (Fig.20B). This indicates that one MycPo molecule is able to bind to another untagged Po molecule homophilically, and that the myc-tag does not separate the MycPo-expressing cells from the untagged, wild type Po-expressing cells in adhesion. Therefore, we conclude that attachment of a myc-tag to the N-terminus of Po protein did not effect the adhesion of Po. The myc-tagged, wild type Po could now be used for the co-transfection experiments with the mutated Po.



B.

Fig.20: A. Aggregation properties of MycPo-expressing cells. A single cell suspension of cells expressing either control transfected cells (control), untagged RPo (WTPo), or myc-tagged Rpo (MycPo) was allowed to aggregate. Samples were withdrawn at various intervals and examined under microscope and counted in a Coulter counter for total particle number. The percentage drop in total particle number ± S.E. was plotted against time. B. Mixed aggregation assay. WTPo-expressing cells and MycPo-expressing cells were labeled with the lipophilic dye diO (green) and diI (red) respectively. These red and green cells formed coaggregates when they were mixed.

## 2.2. Co-expression of wild type Po and mutated Po in CHO cells

Myc-tagged, wild type Po cDNA on plasmid SJL was co-transfected with mutated Po cDNA (hPoS34C, rPoN93A, hPoD61E, hPoK67E or hPoS34Δ) into CHO cells in a molar ratio of 1:1. After G418 selection, the cells were expanded and FACS-sorted after labelling with myc antibody and fluorescence-conjugated secondary antibodies. A population of cells, comprising about 80% of the brightest of staining, was collected and amplified with MTX. The cell line was then examined for co-expression of MycPo and mutated Po. Five cell lines were named as follows. A cell line co-expressing wild type Po and PoS34C was designated as MycPo/PoS34C (Fig.21A, lane e and f). A

Cell line co-expressing wild type Po and PoN93A was designated as MycPo/PoN93A (Fig.21A, lane g and h). A cell line co-expressing wild type Po and PoD61E was designated as MycPo/PoD61E (Fig.21B, lane m and n). A cell line co-expressing wild type Po and PoK67E was designated as MycPo/PoK67E (Fig.21B, lane k and l). A cell line co-expressing wild type Po and PoS34Δ was designated as MycPo/PoS34Δ (Fig.21C, lane 3). Since there is a small molecular weight difference between myc-tagged and untagged Po, two separate bands were observed, as expected, after Western blotting. The molecular weight of myc-tagged, wild type Po is about 31.5 kd while mutated PoS34C, PoD61E, PoK67E and PoS34Δ are all about 30 kd. Staining of MycPo/PoS34Δ cell lysates with myc antibody confirmed that the upper band was MycPo (Fig.21C). To determine if these proteins were glycosylated, co-expressors MycPo/PoS34C, MycPo/PoN93A, MycPo/PoD61E and MycPo/PoK67E were treated with the enzyme, PNGase F which cleaves all N-linked carbohydrates (Fig.21A and B, "+" lanes). After digestion with PNGase F, the molecular weight of all the Po proteins was decreased by about 6% as would be expected. PoN93A, as the mutation destroyed the sugar attachment site, had a molecular weight of 28 kd which did not change after the PNGase F treatment (Fig.21, lane g and h). Although co-expressor MycPo/PoS34Δ was not treated with the deglycosylase, from the molecular weight presented by the Po proteins, it is probable that they are glycosylated (Fig.21C). Figure 21 also shows that there is a comparable amount of wild type Po and mutated Po in MycPo/PoD61E and MycPo/PoK67E, while the expression of wild type Po is slightly higher than that of mutated Po in MycPo/PoS34C, MycPo/N93A and MycPo/PoS34Δ.

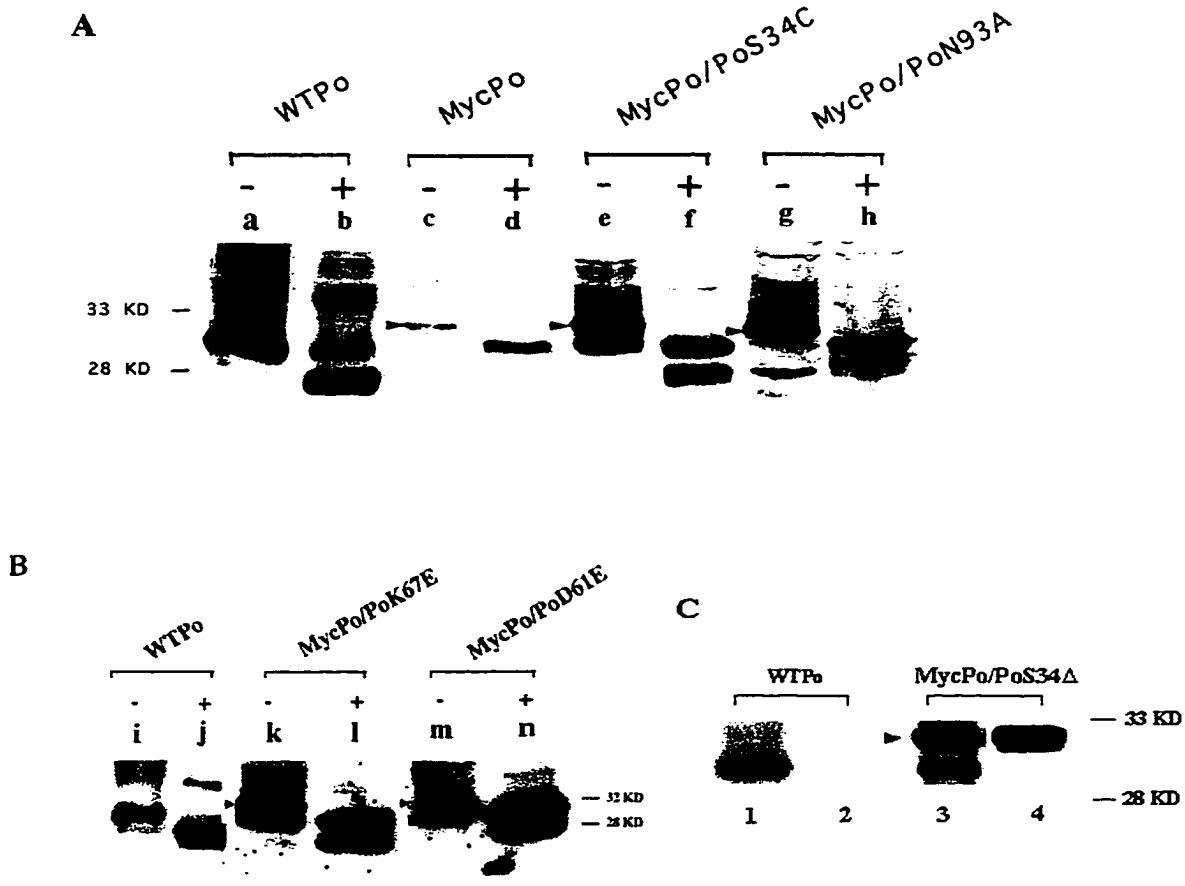
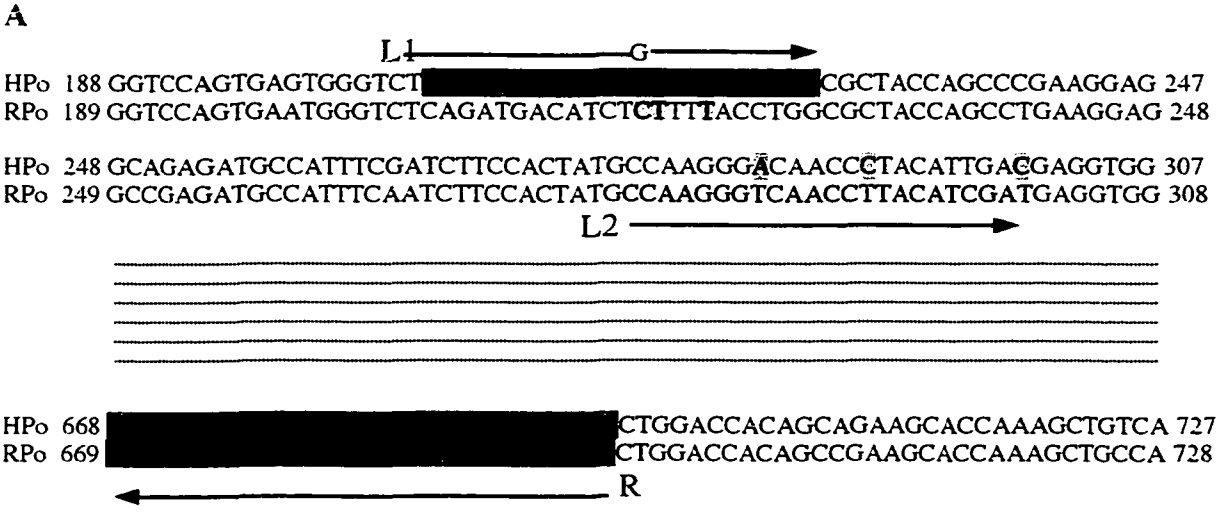



Fig.21: Western blot immunostained for Po of cells expressing wild type Po only (A: lanes a & b; B: lanes I & j; C: lane 1), MycPo only (A: lanes c & d) or MycPo and mutated Po (lanes e-h, k-n and 3). Cell lysates were treated with (+) or without (-) PNGase F as indicated. C: lanes 2 and 4 were immunostained for anti-myc. Arrowheads refer to the position of MycPo. Bars indicate molecular weight standards.

Co-expression of both wild type Po and mutated Po was also examined for MycPo/PoD61E and MycPo/PoK67E at the mRNA level. This was carried out by RT-

PCR. Total RNA was first isolated from the cell lysates and transcribed into cDNA by priming with Oligo (dT). Then Po cDNA was amplified using Po-specific sequences. Since MycPo is expressed from rat Po cDNA while mutated Po is expressed from human Po cDNA, based on the species difference between the two types of Po cDNA, it is possible to detect each Po cDNA individually. Previously, it was shown that primers used for detection of Po mRNA in hPoY125stop or hPoY152stop failed to amplify rat Po cDNA reversibly transcribed from its mRNA (Fig.16: Fig.22B, lane 5 and 7). Therefore this set of primers, namely L1 and R (Fig.22A), was used for distinct detection of mutated human Po cDNA in co-expressors. Another set of primers, namely L2 and R (Fig.22A), was designed and shown to detect only rat and not human Po cDNA (Fig.22B, lane 6 and 8). Using these two sets of primers, RT-PCR was carried out. The results shows that the mRNA from both wild type rat Po and mutated human Po DNA were transcribed in the co-expressors (Fig.22B, lane 1 and 2, lane 3 and 4).



B

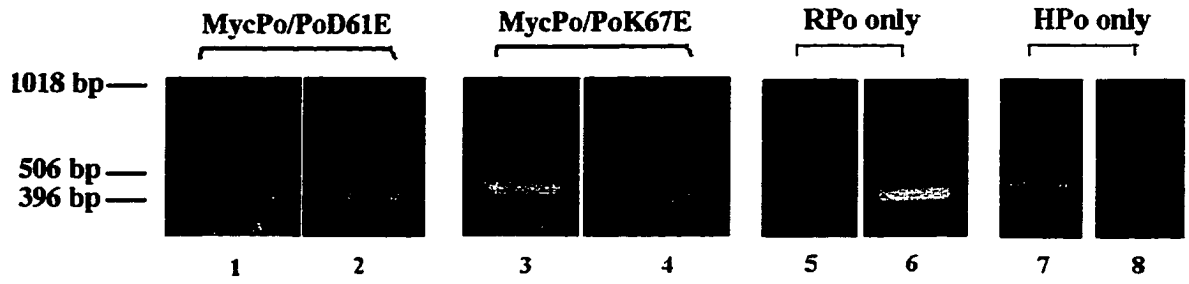



Fig.22: Detection of Po mRNA using RT-PCR. Lanes 1, 3, 5 and 7 are probed with a pair of primers L1 and R, designed against human Po cDNA sequence, while lanes 2, 4, 6 and 8 probed with primers of L2 and R against rat Po cDNA sequence (panel A). Under contingent conditions, primers of L1 and R only amplify human Po cDNA expressed by HPo only cells (lane 7), not rat Po cDNA expressed by RPo only cells (lane 5); while primers of L2 and R only amplify rat Po cDNA (lane 6), not human Po cDNA (lane 7). Both human- (lane 1 or 3) and rat- Po cDNA (lane 2 or 4) are detected by the above sets of primers in co-transfected mycPo/PoD61E (lane 1 and 2) and mycPo/PoK67E (lane 3 and 4).

## 2.3. Surface detection of Po

Two co-expressors, MycPo/PoS34C and MycPo/N93A, were studied further for surface expression of these Po proteins. Surface proteins were labelled with biotin and subjected to two successive immunoprecipitations. The first immunoprecipitation was carried out with myc antibody, then after removal of myc precipitates, the second precipitation was carried out with Po-Ab. The immunoprecipitates were deglycosylated to aid in detection and quantitation. Biotin-binding streptavidin-HRP and an enhanced chemiluminescence (ECL) system were used for detection. Since the cell membrane is impermeable to water-soluble biotin, any labelled protein must be on the cell surface. It was shown that both wild type and mutated Po proteins had reached the cell surface.

Furthermore, the myc-tagged, wild type Po precipitated by myc antibody (Fig.23, lane 5 and 7) was in comparable amount with the mutated Po precipitated by Po antibody (Fig.23, lane 6 and 8). In contrast, for the cells expressing only the wild type Po, only the wild type Po was detected in second immunoprecipitation (Fig.23, lane 3 and 4).

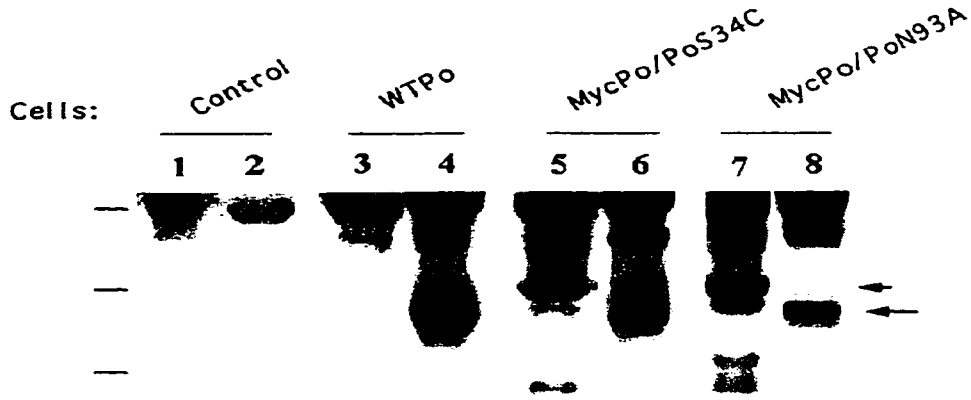



Fig.23: Immunoprecipitation of Po from controls or co-expressing cells that are surface labeled with biotin. Intact cells were covalently labeled with biotin, solubilized, and mycPo was immunoprecipitated with a monoclonal myc antibody (lanes 1, 3, 5, and 7). After removal of myc-precipitates, Po was immunoprecipitated with a Po polyclonal antibody (lanes 2, 4, 6, and 8). Precipitated proteins were deglycosylated, separated by SDS-PAGE, and the biotinylated proteins were visualized with a streptavidin conjugated with HRP and a chemiluminescence method of detection. (Lanes 1 and 2), control cells; (lanes 3 and 4), cells expressing only wild type Po; (lane 5 and 6), cells expressing both wild type and PoS34C; (lanes 7 and 8), cells expressing both wild type and PoN93A. Short arrow refers to myc-tagged, wild type Po, long arrow refers to untagged Po or its mutants. Bars indicate molecular weights as follow: 45, 31 and 21.5 KD.

Surface expression of Po in four co-expressors was also visualized by dual immunofluorescent staining (Fig.24). Unpermeablized, fixed cells were incubated with both an anti-myc (mouse) antibody and a Po -antibody (rabbit), and stained with a FITC-conjugated goat anti-mouse Ig and a phycoprobe-conjugated goat anti-rabbit Ig. Myc-

antibody only stained the co-expressors but not the control, expressing only the untagged, wild type Po (Fig.24, panel A). The intensity of staining of each co-expressor with Po-antibody appeared almost the same as the control cells (Fig.24, panel B). This indicates that there are approximately equivalent amounts of Po expressed on the cell surface in the co-expressors as in the control, expressing only the wild type Po.

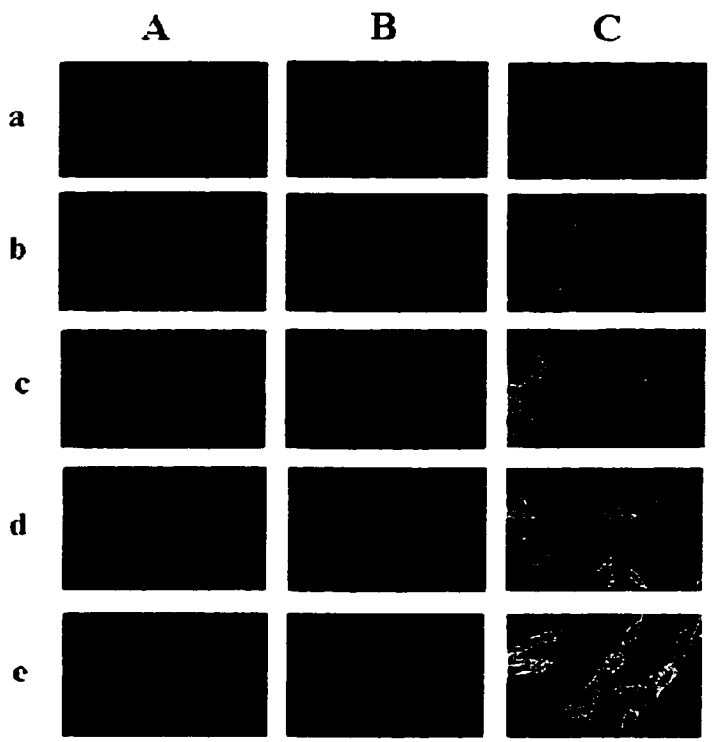



Fig.24: Surface immunofluorescence staining of Po on fixed CHO cells. Transfected cells were fixed and immunostained for myc (panel A) or Po (panel B) using anti-myc or anti-Po antibodies. Fluorescence dyes, FITC (green) or phycoprobe (red) were brought in by a second antibody and the cells were viewed with a fluorescence microscope. a. Cells expressing wild type Po; b, cells expressing mycPo/PoS34C; c. cells expressing mycPo/PoN93A; d, cells expressing mycPo/PoD61E; d, cells expressing mycPo/PoK67E.

## 2.4. Adhesion assay of cells co-expressing wild type and mutated Po

After assessing the expression level of both wild type Po and mutated Po in the co-expressing cell lines, the adhesive properties of each co-expressor were compared to those of the cells expressing only wild type Po in the aggregation/adhesion assay. After incubation of a single-cell suspension for 60 min, none of the coexpressors MycPo/PoS34C, MycPo/PoN93A, MycPo/PoD61E had formed aggregates (Fig.25b, c, d). Indeed, they were indistinguishable from the control transfected CHO cells that did not express Po (data not shown). In the same time, the co-expressors MycPo/PoK67E and MycPo/PoS34Δ had formed some aggregates, but they were both small in size and highly sensitive to agitation, in that moderate shaking broke up these aggregates (Fig.25e, f). In sharp contrast, as we have shown before, by 60 min, the cells that expressed only the wild type Po had formed large aggregates of hundreds of cells (Fig.25a).

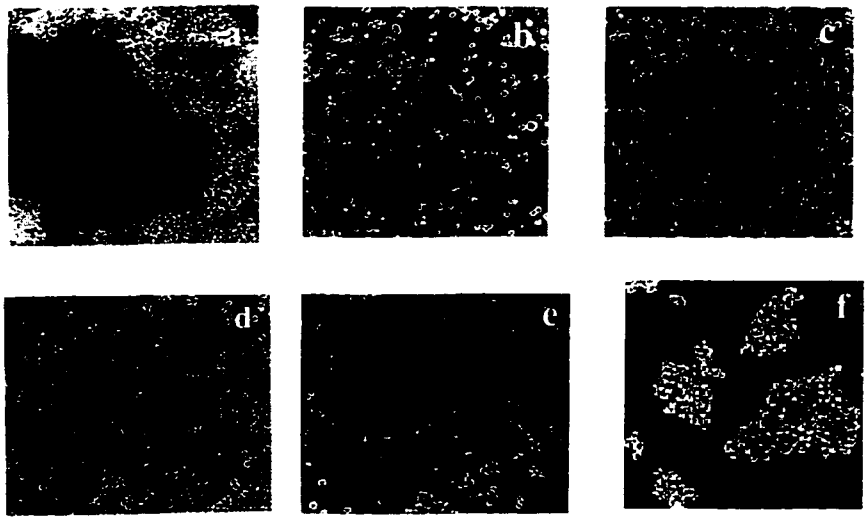



Fig.25: Microscopic view of withdrawn samples after 60 min incubation in aggregation assay.
a: cells expressing with type Po only;
b: MycPo/PoS34C;
c: MycPo/PoN93A;
d: MycPo/PoD61E;
e: MycPo/PoK67E;
f: MycPo/PoS34Δ.

Along with making microscopic observations, the total particle number for those cell lines was periodically counted during incubation. Figure 26 shows the quantitative results from each separately performed aggregation assay in which the percentage drop in the total particle number for each co-expressor was plotted against time. In an adhesion assay with co-expressor MycPo/PoS34C after 60 minute incubation, the total particle number of cells expressing only wild type Po dropped to about 13% of the starting number, while the total particle number of MycPo/PoS34C cells dropped to only about 70% in the same time (Fig.26A). With co-expressor MycPo/PoN93A, its total particle number dropped to about 55% of the starting number by 60 minute, while the total particle number of cells expressing only wild type Po dropped to about 18% in the same time (Fig.26B). In another adhesion assay, by 60 minutes, the total particle number of cells expressing only wild type Po dropped to about 16% of the staring number. In contrast, the total particle number of cells expressing MycPo/PoD61E and MycPo/PoK67E dropped to about 49% and 38%, respectively (Fig.26C). As for another co-expressor MycPo/PoS34Δ, its total particle number had dropped to 28% in 60 minutes, which falls between those for the wild type Po-expressing cells and the control cells (Fig.26D). If the results in each adhesion assay are presented as a percentage of specific adhesion after a 60-min incubation, with 100% adhesion taken as adhesion of the cells that expressed only wild type Po, the specific adhesion for each co-expressor can be plotted on a same scale. This is presented in Figure 27. It is shown that co-expressor MycPo/Po34C is the least adhesive, followed by co-expressors MycPo/PoN93A and MycPo/PoD61E, which are about the same. The other two co-expressors MycPo/K67E

and MycPo/PoS34Δ have exhibited moderate adhesions, which are in accord with the microscopic observation. Taken together, these results suggest that adhesion of wild type Po is blocked completely by the presence of mutated PoS34C, PoN93A or PoD61E; or partially by the presence of mutated PoK67E and PoS34Δ.

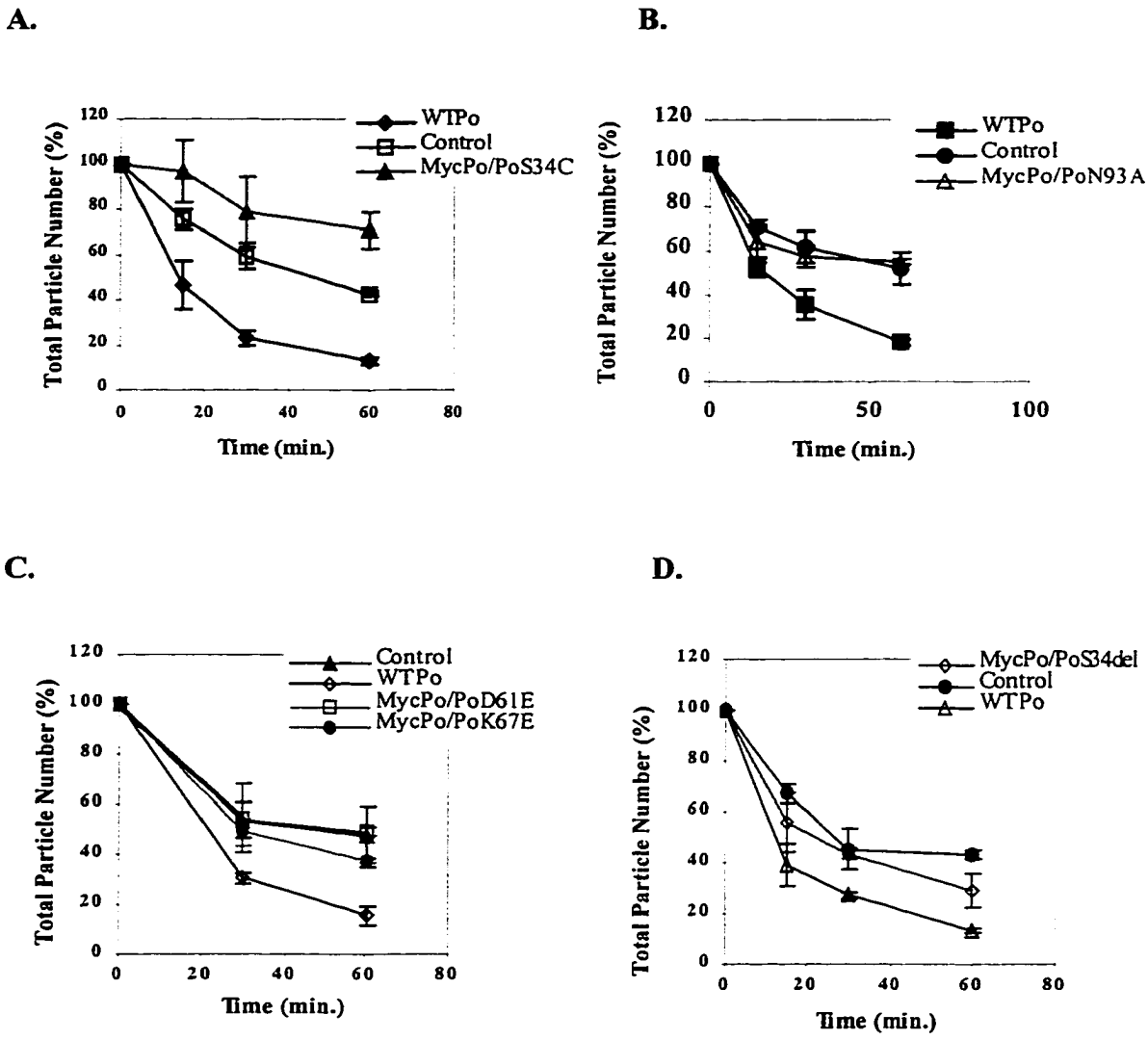



Fig.26: Aggregation properties of cells co-expressing wild type and mutated Po. In the assays, a single-cell suspension of cells expressing MycPo/PoS34C (A), MycPo/N93A (B), MycPo/PoD61E and MycPo/PoK67E (C), or MycPo/PoS34Δ (D) were allowed to aggregate in comparison with the cells expressing only wild type Po or the control. Samples were withdrawn at intervals and examined under the microscope (figure 21) after 60 min aggregation and the total particle number was counted in a Coulter counter. Total particle number in percentage was plotted against time.

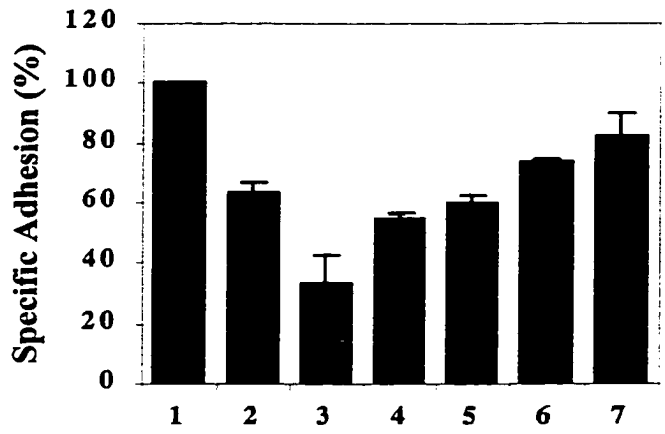



Fig.27: Comparison of specific adhesion of all co-expressors. Specific binding was calculated as the percentage drop in total particle number of the cells expressing only the wild type Po was taken as 100%. Column 1, cells expressing only the wild type Po; 2, the control; 3, MycPo/PoS34C; 4, MycPo/PoN93A; 5, MycPo/PoD61E; 6, MycPo/PoK67E; 7. MycPo/PoS34Δ. Results presented are specific adhesion (+/-S.E.) after 60 min incubation.

## 3. Summary

1) Myc-tagged wild type Po is as adhesive as untagged, wild type Po.

2) Both myc-tagged, wild type Po and mutated Po proteins are expressed in the co-transfected cells, as determined by molecular difference after Western blotting.

3) In co-expressors, both myc-tagged, wild type Po and mutated Po were glycosylated to the same extent.

4) Co-expressors showed sufficient levels of wild type Po at the cell surface to be adhesive if expressed alone.

5) Myc-tagged, wild type Po and mutated Po expressed on the cell surface are in comparable amounts.

6) As assessed by the adhesion assay, mutation of Po by S34C, N93A or D61E had a dominant-negative effect on the wild type Po, while mutation of Po by K67E or S34Δ had a partial dominant-negative effect.

# Chapter V

# General Discussion

Protein zero (Po), an integral membrane glycoprotein synthesized by Schwann cells, is the major glycoprotein of peripheral nerve myelin. In human, the genetic defects in the Po gene (MPZ) have been associated with a wide spectrum of hereditary motor and sensory peripheral neuropathies (HMSN), ranging from the milder CMT (Charcot-Marie-Tooth) 1B to the more severe DSS (Dejerine-Sottas Syndrome) and CH (Congenital hypomyelination) (Patel and Lupski, 1994; Chance and Fischbeck, 1994; Warner *et al.*, 1996). To date, more than 57 different mutations in Po, which include deletions, insertions, missense and nonsense mutations, have been identified in these HMSN patients (Nelis *et al.*, 1999). However, despite the rapid progress in genetic identification of the Po mutations, little is known about the mechanism whereby the mutations in the gene of Po cause the heterogeneous forms of the disease and how the phenotype is correlated to the genotype.

One possible mechanism whereby a mutation in Po could cause the demyelinating disease, is the reduction in Po's gene dosage. Most affected individuals are heterozygotes for the defective gene. That is to say, half of the Po protein is normal and half is aberrant. As a consequence, the level of wild type Po protein in these patients will be half of that of the normal individuals. If a threshold level of wild type Po in the cell membrane is required for the maintenance of myelin and half of the normal dose is below that level, insufficient wild type Po in these patients is the reason for the disease. If presumably, 50% of the Po protein produced by the mutated allele is disrupted to such an extent that it does not reach the cell surface, this scenario will resemble those of the heterozygous Po knock-out mice. These $Po^{+/-}$ mice show normal myelination until 4 months of age, and then develop a mild form of demyelination (Martini *et al.*, 1995). In contrast, the

homozygous Po knock-out mice have first signs of demyelination at 4 days of age (Giese *et al.*, 1992; Martini *et al.*, 1995). However, what is different from the Po heterozygous null mice is that the mutated allele in heterozygous patients could produce an aberrant Po that reaches the surface. In that case, if the mutated Po protein is not adhesive and has no effect on the functioning of the wild type Po, the dose effect could still apply, as the disease phenotype would sorely result from a decrease in functional Po in myelin. The dosage sensitivity of Po gene is also shown in rare cases of HMSN with homozygous mutations in MPZ. In two pedigrees of HMSN (Ikegami *et al.*, 1996; Warner *et al.*, 1996), the parents which are heterozygous for the Po mutations presented as mild cases of CMT1B, while the children, homozygous for the same mutations, have the more severe DSS. In one case, a 1 bp deletion leads to a premature stop that predicts a truncated protein of only 87 amino acids (Warner *et al.*, 1996). It is presumed that this truncated protein is degraded and never reaches the membrane. Thus, the variation in the phenotypic presentation of these heterozygous and homozygous mutations of MPZ mutations is similar to those of heterozygous and homozygous mice for the null mutation of Po gene (Giese *et al.*, 1992; Martini *et al.*, 1995).

A gene dosage effect certainly applies to another group of HMNS patients involving PMP22 gene defects. A tandem duplication in chromosome 17p11.2-12 resulting in trisomic overexpression of PMP22 gene is responsible for disease in most CMT1A patients. Deletion in the same region causing monosomic underexpression of PMP22 gene is the reason for some HNPP patients. So a tight control on the expression level of myelin genes must be important for normal function of myelin.

The reduced Po gene dosage effect could explain the very mild CMT1B. The best example is the pedigree we just mentioned above. The heterozygous parents with a 1 bp deletion in Po have a symptom so mild that they are not clinically assessed prior to the evaluation of their children (Warner *et al.*, 1996). However, as to more severe CMT and DSS, another possible mechanism whereby a mutation in the Po gene would result in the demyelinating disease, would take effect. In this case, it is more likely the mutated Po protein reaches myelin, but in addition to being non-adhesive, interferes with the functioning of normal Po protein. That is to say, the presence of the aberrant Po protein from the mutated allele may have a dominant-negative effect on the wild type Po. Further, a rather broad range in the clinical severity suggests that different degrees of the dominant-negative effects may exist.

The *in vitro* transfection/aggregation system provides us an excellent tool to test which possible cause of demylination is likely to be true for each Po mutation associated with CMT1B. In this study, we have chosen 4 missense (S34C, D61E, K67E and N93A), 1 deletion (S34Δ) and 2 nonsense (Y125 and Y152) mutations of Po to study further. By making permanently transfected cell lines for each mutated Po and by co-expression of the mutated Po with wild type Po also in permanently transfected cell lines, the ability of each mutant Po to reach the cell surface, to mediate adhesion and to have a dominant-negative effect on the wild type Po was assessed (see Table 2).

Table 2: Summary of experimental results.

| Mutations | Surface Expression | Adhesive alone | Dominant/Negative if coexpressed |
|---|---|---|---|
| **S34C** | + | - | +++ |
| **S34Δ** | + | - | + |
| **D61E** | + | - | ++ |
| **K67E** | + | - | + |
| **N93A** | + | - | ++ |
| **Y125Stop** | - | N/A | N/A |
| **Y152Stop** | - | N/A | N/A |

It was found that (1) for nonsense mutation of either Y125 or Y152, Po mRNA was expressed as determined by RT-PCR analysis, but Po protein was undetectable as assessed by Western blot analysis. (2) For the other four mutations (S34C, S34Δ, D61E K67E and N93A), Po protein was expressed, reached the cell surface, and was glycosylated (except N93A) to the same extent as the wild type Po. This suggests that these mutations do not affect normal synthesis and processing of Po. Furthermore, because these mutated Po proteins are still glycosylated and are still recognized by Po antibody as well, the overall structure of Po is unlikely to be greatly altered. However, all these mutations result in loss-of-function as these Po mutants fail to mediate cell-cell adhesion. Although we have not observed it in these studies, it is possible for some Po mutations that Po protein is expressed but remains in the cytoplasm and does not reach the cell surface. The accumulation of mutant proteins may have a detrimental or toxic effect on the cell. This situation is described for some mutations in other myelin membrane proteins such as PLP (Gow and Lazzarini, 1996) and Cx32 (see review Scherer *et al.*, 1997).

Po protein contains an extracellular domain with an Ig-like motif (residues 1-124), a single membrane-spanning domain (residues 125-150) and a basic intracellular domain (residues 151-219). The transmembrane domain is bound by the basic residue Arg at 124 and also at 151. Therefore, Tyr125 is the first residue of the transmembrane segment and Tyr152 is the second residue into the cytoplasmic domain. The Tyr152stop mutation results in deletion of the entire intracellular domain, while the Tyr125stop mutation will abolish both the transmembrane and intracellular domains completely. Because no expression of either of these two truncated proteins was detected in CHO cells, while the entire Po mRNA was present, it is suggested that the mutated protein is rapidly degraded, and this degradation must be a direct result of truncation of the entire intracellular domain. In a previous study, Po truncated by 59 amino acids from the cytoplasmic domain was expressed on the cell surface (Wong and Filbin, 1994). This suggests that the nine amino acids (YCTLRRQRR) adjacent to the membrane which are missing in Tyr152stop, are necessary for stabilization of the polypeptide and targeting it to the cell membrane. When Po protein is synthesized on the rough endoplasmic reticulum (RER), the extracellular domain of Po enters the lumen of the RER while the transmembrane domain is anchored to the RER membrane (D'Urso *et al.*, 1990). It is possible that those nine amino acids may help to strengthen the anchorage and guide the protein through the sorting process to the cell membrane. Absence of those sequences may signal degradation. Interestingly, Cys153, one of those 9 amino acids, is an acetylation site for wild type Po (Agrawal *et al.*, 1983; Bizzozero *et al.*, 1994). It is suggested that fatty acid attachment may stabilize Po at the plasma membrane (Bizzozero *et al.*, 1994; Filbin and Tennekoon, 1992). It is found that although Po point-mutated at Cys153 reached the

membrane, it had a faster turn-over than wild type Po and also was not adhesive (Gao and Filbin, unpublished data). Therefore, the absence of Cys153 could contribute to the unstable behaviour of Y125stop and Y152stop. However, absence of Cys153 is not the only factor causing lack of surface expression and instability because Po mutated at Cys153 but with all other cytoplasmic domain amino acids intact, did reach the membrane.

The other five mutations studied are located in the extracellular domain of Po (Po-ED), where most of the other CMT1B mutations occur. Since the mutated amino acids are scattered around the whole extracellular domain, it is unlikely that all of them are directly involved in adhesion. Some mutations may cause a loss-of-function by disrupting the conformation of the Po molecule. One such example is the mutation that disrupts the disulfide bond of Po's Ig domain (Zhang and Filbin, 1994; 1997). The disulfide bond, formed between Cys21 and Cys98 of Po, is believed to stabilize the compaction and orientation of a pair of apposed β-sheets within the Ig-domain. Disruption of the disulfide bond is more likely to cause a conformational change, which then indirectly affects adhesion. It was shown that Po mutated at Cys21 could no longer adhere (Zhang and Filbin, 1994). Furthermore, a mutation at Cys98 was recently found in one pedigree of DSS patients (Haites *et al.*, 1998; Fabrizi *et al.*, 1999). If the mutated protein is mis-folded to such an extent, it could be held in the RER, or degraded before presented on the membrane. However, as Cys21-mutated Po was still present on the cell membrane and recognized by a polyclonal Po antibody (Zhang and Filbin, 1994), the change in protein conformation must not be that drastic. Nevertheless, a conformation-

dependent monoclonal antibody (mAb) will be useful in determining whether a site-directed mutation effects function of the protein directly or indirectly. Such applications have helped to localize the putative sialic acid-binding site on several sialoadhesins including sialoadhesin, CD22 and MAG (Vinson *et al.*, 1996; Van der Merwe *et al.*, 1996; Tang *et al.*, 1997). Since such conformation-dependent monoclonal Po antibody is not developed yet, whether the five mutations we studied here (S34C, S34Δ, D61E K67E and N93A) cause a conformational change or affect adhesion directly may not be easily answered without examining the 3D structure of Po.

Prior to when the crystal structure of Po-ED was solved, a molecular model for Po-ED was proposed (Wells *et al.*, 1993). It was based on the similarity of Po-ED in both sequence length and secondary structure to the $V_H$ domain of the phosphocholine-binding mouse immunoglobulin M603 (Inouye and Kirschner, 1991), whose structure had been determined crystallographically (Padlan, 1977; Satow *et al.*, 1986). Although this model and the later crystal structure are based on analysis of the rat Po-ED sequence, the structure of human Po-ED are predicted to be the same as both Po-ED sequences differ only at two positions (Tyr10 and Ser77 for rat versus His10 and Arg77 for human). The three-dimensional structure of Po-ED predicted in this model (Wells *et al.*, 1993) is mostly in accord with the crystal structure for Po-ED later solved by Shapiro *et al.* (1996) except for slight differences. For example, the ten β-strands of Po-ED in the model correspond closely to those deduced from the X-ray result (i.e. A, A′≅1; B≅2; C≅3; C′≅4; C″≅5; D≅6; E≅7; F≅8; G≅9, 10). The residues Ser34 and Lys67 are predicted to be on β-strand 3 and on the intervening loop 5-6, respectively, which matches the crystal analysis.

Asp61 and Asn93, predicted to be on β-strand 5 and at the bottom-end of loop 7-8 by Wells *et al.*, is located at the beginning of loop C''-D and beginning of β-strand F by the crystal analysis, respectively. However, there is somewhat difference on interpretation of how the Po-ED is oriented and assembled at the cell surface, as proposed by the two models.

According to the model of Wells *et al.*, the long axis of Po-ED molecule is perpendicular to the lipid bilayer membrane. The apposed Po-ED would align end-to-end during myelination, and then slide over each other and overlap nearly fully after compaction in the native state. Two of the side faces of Po-ED that show charge complementarity could account for the homophilic interactions of Po-ED from apposed membranes in the native state. In this model the charged residues, Asp61 and Lys67, are located on one such face and directly involved in these electrostatic interactions. It is predicted that the conservative mutation Asp61→Glu would increase the length of the side chain by –CH2–, hence affect the interaction between the two faces if distance is crucial in addition to charge. The mutation of Lys67→Glu would clearly disrupt the charge complementarity between the two faces (Kirschner and Saavedra, 1994). Therefore, the loss of adhesion by mutation at Asp61 or Lys67 of Po is probably due to disruption of Po-Po interaction directly. In this model, Ser34 may not directly participate in the homophilic interaction. Deletion of Ser34 which result in a shorter β-strand and an accommodated rather long loops flanking the β-strand, may have a detrimental effect on the conformation of the protein, which in turn effect adhesion. The Ser34→Cys mutation, predicted to result in inappropriate formation of either intra- or intermolecular

disulfide bonds (Kirschner and Saavedra, 1994), would definitely effect both the protein conformation and the protein-protein interaction. Another mutation at Ser34, replaced by Phe (Blanquet-Grossard *et al.*, 1995), would also likely cause a conformational change. Therefore, Ser34 may be a key residue in regard to the protein conformation. However, as the mutated protein was still able to be glycosylated and also reached the membrane, it indicates that the original structure is somewhat retained by the mutated protein. Our previous study showed that Po must be glycosylated for adhesion to occur (Filbin and Tennekoon, 1991, 1993). Removal of oligosaccharide attachment by mutation Asn93→Ala, or changing the sugar from complex to high-mannose form abolished the adhesive properties of Po. However, the soluble Po-ED, which was expressed in bacteria and hence did not carry carbohydrates, retained strong homophilic binding capacity (Griffith *et al.*, 1992). Taking these results together, we suggested that, rather than being directly involved in adhesion, the carbohydrate of Po plays an important role in positioning the Ig-domain of Po in an optimal orientation relative to the membrane to interact with another Po molecule in the opposing membrane (Filbin *et al.*, 1996). The model of Wells *et al.* supported this suggestion. Since Asn93 is located at the bottom of the Ig-domain in the model, it is predicted that the carbohydrate would ensure an appropriate orientation of the protein at the membrane surface. If the carbohydrate is removed, the Ig-domain would collapse back to the membrane and fail to interact with another Po molecule (Wells *et al.*, 1993).

On the other hand, based on the crystallographic data, Shapiro *et al.* (1996) proposed a tetrameric Po network at the membrane surface. According to his model, four

molecules (protomers) of Po-ED from the same membrane plane form a tetramer, with the long axis of each protomer tilted by 45° with respect to the membrane. Each tetramer would be able to interact with another four tetramers from apposed membrane. As the dimeric association between or within tetramers is generally weak, the face to face interaction predicted by Wells *et al.* (1993) is not favoured. Instead the loop to loop interactions are mostly used. It is predicted that the B–C loop (at top) of one molecule would interact with the C″–D and E–F loops (at bottom) on the next molecule from the same plane. Since Asp61 and Lys67 are located on the C″–D loop, their side chains probably participate in this interaction. Therefore, the mutations of Asp61→Glu and Lys67→Glu would likely impair the tetramer formation. Once again, Asp61 and Lys67 are predicted to be directly involved in the homophilic interaction of Po. Wells *et al.*'s predication about location of Ser34 in the middle of a β-strand is confirmed by the crystallographic analysis. Furthermore, it is predicted that substitution of Ser34 with Cys will produce outwardingly pointing thiols, which tend to form abnormal disulfide aggregates in the extracellular space. In the crystal structure, Asn93 is at the beginning of the F strand near the membrane-proximal base of the molecule, which supports our prediction about the role of the carbohydrate chain in adhesion.

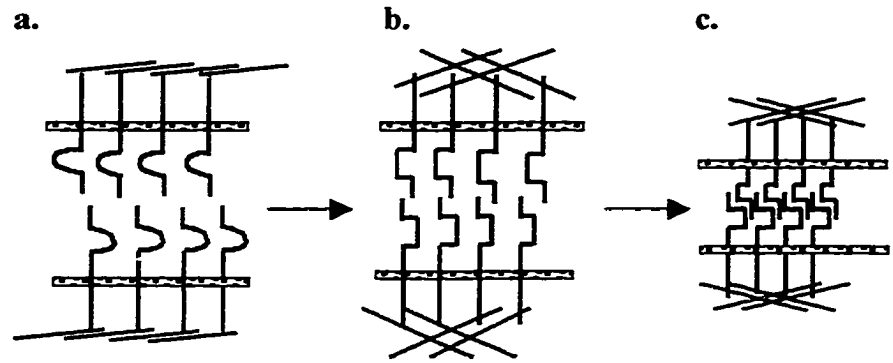
All the CMT patients carrying any one of these seven mutations (S34C, S34Δ, D61E, K67E, N93A, Ty125stop and Tyr152stop) are heterozygotes, which means that the normal allele for the Po gene co-exists with the mutant allele. A dose reduction in the Po gene appears to be the reason for the Tyr125stop or Tyr152stop mutations to cause the demyelinating disease, as neither of these mutated proteins reaches the membrane,

resulting only 50% of normal levels of wild type Po in these patients. However, the other five mutated Po proteins are expressed on the cell membrane but failed to adhere in adhesion assay. Their possible dominant-negative effects on wild type Po were assessed by co-transfection of the cDNA of each these mutated Po with the wild type Po into CHO cells. It was shown that all five mutated Po proteins exhibited mild or significant dominant-negative effects on the wild type Po (see Table 2). Similar effects have also been shown for Po truncated in the cytoplasmic domain (Wong and Filbin, 1996) and Po mutated at Cys21 (Zhang and Filbin, 1998).

Previously we suggested that Po must cluster in the same membrane, *cis*-interactions, to allow adhesion to occur. Furthermore, results suggest that clustering is mediated through the interaction between the cytoplasmic domain of Po and the cytoskeleton (Wong and Filbin, 1994 and 1996). The idea of clustering is in agreement with the Po tetramer model proposed by Shapiro *et al.* (1996). If a critical number of adhesive, wild type Po molecules in each cluster, presumably a tetramer, is required for effective adhesion to occur, it is likely that the non-adhesive, mutated Po (Po S34C, S34Δ, D61E, K67E and N93A) when co-expressed with the wild type Po, could join the tetramer and dilute out the number of the wild type Po's in the tetramer. As a result, the sum affinity per tetramer would be reduced to such an extent that effective adhesion between opposing tetramers would no longer be sustained, namely a dominant-negative effect (Fig.28).

Another possible mechanism whereby the mutated Po exerts its dominant-negative effect on the wild type Po is that, instead of creating a tetramer with affinity too

**A. Wild type Po**




**B. Wild type and point-mutated Po**

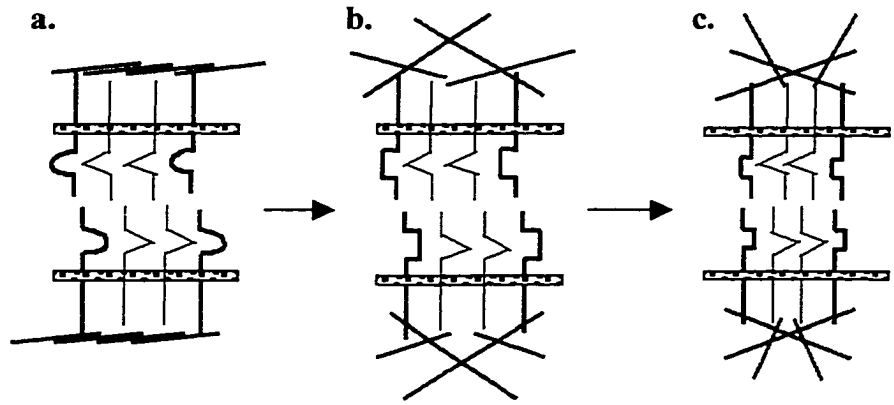



Fig.28: Model of a dominant-negative effect of the point-mutated Po on the adhesion of wild type Po. (A) Interactions of wild type Po when expressed alone. (a) Po is associated with cellular cytoskeleton. During the cell-cell adhesion, an initial low affinity interaction occurs between Po's extracellular domains in the opposing cell membranes and this triggers a change in the interaction of Po's cytoplasmic domain with the cytoskeleton. (b) The cytoskeleton re-organizes and induces clustering of Po molecules and a conformational change in the extracellular domain of Po. (c) Both clustering and conformational change of Po induce a strong adhesion of Po's extracellular domain. The stripped bars represent cell membranes and the grey straight lines represent cytoskeletons. (B) Interactions of wild type Po when co-expressed with the mutated Po. (a) Both wild type (dark) and mutated Po (light) are associated with cytoskeleton. The initial interaction between the extracellular domains of Po in the opposing cell membrane induces a change in the interaction of Po's cytoplasmic domain with the cytoskeleton. (b) The cytoskeleton tries to re-organize, but fails to induce a conformational change in the extracellular domain of mutated Po. (c) The overall adhesiveness is decreased because of the presence of the non-adhesive mutated Po. Adhesion is not strong enough to hold two membranes together.

weak to adhere to the opposing tetramer, the presence of mutated Po molecules may completely prevent formation of Po tetramers. This is a more likely mechanism for the effects of mutated PoD61E and PoK67E, as both Asp61 and Lys67 are proposed to be directly involved in the interactions which hold the homotetramer together. It has been suggested that the affinity of individual adhesion molecules is generally weak but by forming a multimeric network the adhesiveness is strengthened (Van der Merwe and Barclay, 1994; Shapiro *et al.*, 1996). As a result, if the homotetramer is not formed, individual Po molecules with weak affinity will fail to produce homophilic adhesion with an opposing Po molecule.

Although Po must be clustered to allow adhesion, clustering must not be at random. Mutation of S34C produces an outwardly orientated thiol, which has the potential to aggregate more Po molecules by forming intermolecular disulfide bond. However this has a very deleterious effect on the adhesive function of Po protein as this mutation generates the more severe DSS. It would appear that a mutation to Cys leads to a more severe CMT1B or DSS phenotype (Haites *et al.*, 1998; Hayasaka *et al.*, 1993; Sorour *et al.*, 1998; Silander *et al.*, 1998; Bort *et al.*, 1997; Gabreëls-Festen *et al.*, 1996; Warner *et al.*, 1996; Rouger *et al.*, 1996). The only exception, to date, is Tyr53→Cys, which is associated with the milder CMT1B (Himoro *et al.*, 1993). However, as Tyr53 is predicted to be buried in the hydrophobic core of the molecule, the substituted Cys would not be available to form extra disulfide bonds with neighbouring molecules.

Our results show that Po mutated at Ser34, Asp61, Lys67 and Asn93 exert different degrees of dominant-negative effects on the wild type Po. Wild type Po co-

expressed with the mutated PoS34C, was the least adhesive among all the co-expressors. This behaviour correlates with the disease phenotype as the patients with mutation of S34C are diagnosed as DSS, much more severe than CMT1B, with an early onset and extremely slow nerve conduction velocity. This correlation is more obvious when it is compared to another mutation at the same amino acid. Deletion of Ser34, which shows a milder dominant-negative effect, is associated with a mild CMT1B. Surprisingly, a conservative substitution (Asp61→ Glu) causes less adhesion than a dramatic change (Lys67→ Glu). Both amino acids are located on the C''-D loop, this loop and E-F loop are predicted to interact with the B-C loop from the next molecule in the tetramer model (Shapiro *et al.*, 1996). If hydrogen bonding rather than ionic bonding is favoured among the interaction of the side chains from these loops, then the distance between those side chains is more important than the carried charge. Replacement of Asp with Glu will increase the length of the side chain while replacement of Lys with Glu will shorten that. It can be imagined that an extended side chain would push the neighbouring molecule away, which could cause more damage than a shortened side chain. It appears that patients affected by the Asp61Glu mutation have overall more severe symptoms than those affected by Lys67Glu, although both pedigrees are diagnosed as CMT1B. It was shown that in an affected family, 11 out of 13 members had an estimated age of onset below 10 years and nerve conduction velocities in the range of 8 to 15 m/sec (Hayasaka *et al.*, 1993; Bird *et al.*, 1997). Although the severity of the clinical phenotype is largely determined by the location and type of mutation in the Po gene, a definite correlation between the genotype and phenotype is still elusive, as not enough detailed clinical data

is available. Furthermore, it could be difficult, as a certain inter-individual variability in the clinical severity could exist, even among members of the same family. In such cases, background genes or environmental factors may modulate the disease expression. A final solution to that would be creation of heterozygous transgenic mice carrying these mutations. Our CHO expression/adhesion system could be a sensitive subsidiary to predict which mutation might cause the severe type of CMT1B disease.

# Chapter VI

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