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MAPPING THE FUNCTIONAL REGIONS OF THE MYELIN PO PROTEIN'S EXTRACELLULAR DOMAIN

by

KEJIA ZHANG

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

1998

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

MAPPING THE FUNCTIONAL REGIONS OF THE MYELIN PO PROTEIN'S EXTRACELLULAR DOMAIN

by

Kejia Zhang

Advisor: Professor Marie T. Filbin

Po, the most abundant protein of peripheral nervous system (PNS) myelin is a homophilic adhesion molecule and is directly involved in the compaction of PNS myelin. To map more precisely regions of Po's extracellular domain that are critical for adhesion, antibodies against three hydrophilic sequences, Po 38-46, 74-82 and 91-95 and the peptides themselves were assessed for their ability to inhibit the adhesion of Po-expressing CHO cells. Both peptide 91-95 and its antibody completely inhibited the aggregation of Po-expressing cells. In addition, peptide 74-82 and its antibody also inhibited adhesion but not as effectively as peptide 91-95. In contrast, neither peptide 38-46 nor its antibody had any effect on Po-mediated adhesion. This suggests that sequence 91-95 in Po's Ig domain may be directly involved in the adhesion of Po, while the amino acid sequence 74-82 may be partially involved. To study further Po peptide 91-

95, two amino acids, Asp92 and Gly94, conserved in a subset of Ig-like molecules, were replaced, each with a conservative substitution. Unlike the cells expressing the wildtype Po, cells expressing Po mutated at Asp92 and Gly94 did not form large aggregates. This shows that Po mutated at Asp92 and Gly94 is not capable of homophilic adhesion.

To address the role of the disulfide bond in an Ig-like domain, the disulfide bond between Cys21 and Cys98 of Po's Ig-like domain was prevented from forming by replacing Cys21 with alanine. The Cys21-mutated Po cDNA was expressed in CHO cells which, unlike the wildtype Po, failed to adhere. This suggests that the Po protein, when mutated at Cys21, does not behave like a homophilic adhesion molecule, which in turn implies that the formation of an Ig-like domain disulfide bond is essential to the functioning of Po.

To test the possible dominant negative effect of the aberrant Cys21-mutated Po on the adhesion of the wildtype Po, the cDNA of the Cys21-mutated Po was transfected into a CHO cell line already expressing wildtype Po. Two clones, each expressing both Cys21 Po and wildtype Po lost their Po-dependent adhesive properties, while a third clone, expressing only the wildtype Po, retained its adhesive properties. These results suggest that the presence of Cys21-mutated Po abolishes the adhesion of the wildtype Po protein.

DEDICATION

谨献给

两位引导我步入亨特学院并最终完成这篇论文的朋友和同学
小萍和笑英

她们的美好理想和远大前程不幸过早断送于一次意外事故中，
但她们的聪明智慧，热情活力以及对生活的积极进取是我永远取之不尽的
精神源泉

To the memory of my friends and outstanding fellow students, Xiao-ying and Xiao-ping, who led me to Hunter College and to this thesis.

Their dreams for a bright future in America were both tragically cut short by untimely accidental deaths. Their intelligence, enthusiasm, optimism, energy, and will to succeed are an unending source of inspiration to me.

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Most of all, I would like to thank my mentor Professor Marie T. Filbin, for the guidance and scientific training she gave me. Not only does she strive to stay in the forefront of science, but is also keen and generous to share her knowledge and know-how with us. To me, she is always a source of encouragement and support. I admire her dedication and her very real sense of duty to her work.

I am also grateful to our lab neighbor Professor Thomas Schmidt-Glenewinkel, a man who knows and truly enjoys science. He has always been generous in sharing his knowledge and time. Whenever possible, Dr. Schmidt-Glenewinkel went out of his way to help. I think he may be the very best neighbor one can ever find.

I am indebted to my friend Dr. Tao Wang for his time, expertise and generous technical support. Special thanks are also extended to Dr. Yamina Merazga for her major involvement in the work of characterizing Po mutated at residues 92 and 94.

Furthermore, I wish to thank all the students, staff and post-docs in Dr. Filbin's lab; to the old folks Manhar and Gitali; to the enthusiastic gang who are still diligently carrying out Dr. Filbin's saga: Maria Elena, Song, Yingjing, Wenhui; and to the newcomers: Sussana, Chhaya, Ying, Doming and Jane. It has been enjoyable and precious to be with all of them.

Thanks also go to my fellow student Anne Saunders who aided in the earlier days when I was struggling with problems created by my late arrival at school, compounded by language difficulty and a loss of friends. Without her notes and generous help, it would have been impossible for me to handle those courses and also try to take my first level exam after just one semester.

I also present this thesis to my parents Ping and Rui-lian, my sister Yong and my friend Arnie, as a gift of gratitude for their understanding and unquestioning support throughout the years of my Ph.D. candidacy at Hunter College, and as a token of my respect and affection for each of them.

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Introduction

Myelin in the Peripheral Nervous System

The Structure of Myelin

Myelin is a multilayered assembly of membranes wrapped around axons, which functions as an insulator to increase the velocity of the nerve impulse (Rushton, 1951; Ritchie, 1982). In the central nervous system (CNS) myelin is laid down by oligodendrocytes (Bunge et al., 1961, 1962; Peters, 1964), while in the PNS the Schwann cell is responsible for the formation and maintenance of myelin (Peters and Vaughn, 1970; Martin and Webster, 1973). The typical structure of the myelin sheath is formed by repeated spiral wrapping of newly synthesized cell membrane, from the myelinating cells, around axons (Fig. 1). The subsequent compaction of the adjacent lamellae at apposed cytoplasmic leaflets, results in the major dense line, while interactions at the extracellular surface form the less-dense, double intraperiod line as seen under the electron microscope (Robertson, 1955).

The basic structure of CNS and PNS myelin is similar. In a cross-section of myelinated nerve fibers, the compacted myelin sheath possesses a typical periodicity of alternating major dense lines and intraperiod lines. The distance between major dense lines in the PNS is approximately 12.5 nm, while in the CNS it is somewhat smaller at 11.5 nm (Raine, 1984).

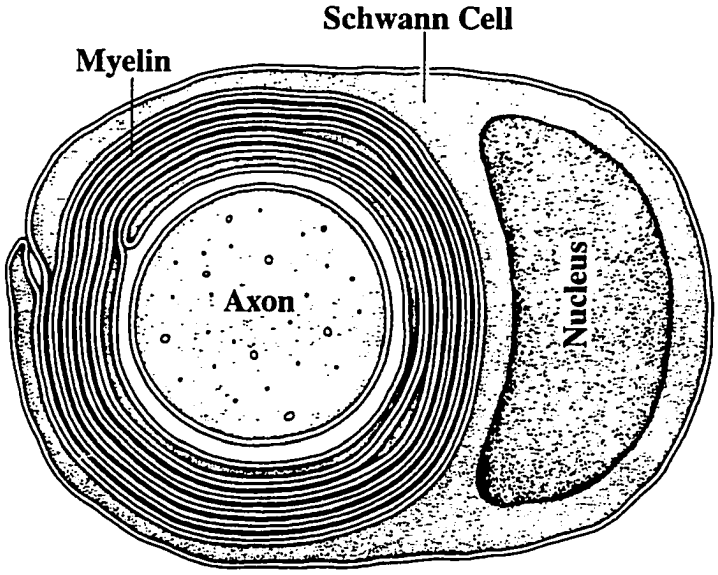


***Figure 1*:** Schematic diagram of PNS myelin sheath and Schwann cell. An axon is myelinated by a Schwann cell with multiple lamellae of plasma membrane. The plasma membrane of the Schwann cell repeatedly wraps around the axon. The subsequent compaction of the adjacent lamellae at the apposed cytoplasmic leaflets results in the thick major dense line, whereas interactions at the extracellular boundary form the less-dense, double intraperiod line as seen under electron microscope. Modified with permission from *Myelin*, P. Morell, ed. (1984), Plenum Publishing Corp., New York, originally created by Morell and Norton (1980).

The myelin sheath is laid down along the length of an axon in segments. Each segment of the myelin sheath is known as an internode. The naked axonal regions between the myelin segments are called the nodes of Ranvier (Raine, 1984). In PNS myelin of mammalians, the internodes are from 200 to over 2000 μm in length (Kirschner et al., 1984). The internodal myelin serves as an insulator for electrical conduction. Therefore, it is only at the nodes of Ranvier that electrical excitement can occur, resulting in the propagation of the nerve impulse in a manner of

jumping from node to node, so-called saltatory conduction. Such saltation, as a result of myelination, increases the conduction velocity of the nerve.

The proper functioning of the nerve is dependent on the maintenance of the integrity of the myelin sheath. This can best be demonstrated from the consequences of demyelination in diseases such as multiple sclerosis in the CNS and Guillain-Barré Syndrome (GBS) in the PNS. Myelin in both the CNS and PNS can be either primarily and selectively affected or, be damaged secondarily to other conditions such as neoplasm, trauma, necrosis and anoxia. Early pathophysiological studies showed that demyelination was associated with slowing of conduction and conduction block (McDonald, 1963; McDonald, 1970a, b). Therefore, understanding myelin at the cellular and molecular level is pertinent to prevention and treatment of demyelinating diseases.

Myelination of the Peripheral Nervous System

Myelin accounts for 50% of brain white matter chemical composition and possibly an even larger percentage of myelinated peripheral nerves (Everly et al., 1973; Kitamura et al., 1976). In the PNS, the Schwann cell is responsible for the formation of myelin. Schwann cells are derived from the neural crest (Harrison, 1924). In rat sciatic nerve, by embryonic day 14 to 15 (E14-E15), precursors of Schwann cells can be first

identified and seen in the nerve shortly after the outgrowth of axons. These precursor Schwann cells move along as axons grow toward their targets (Peters and Muir, 1959; Gamble and Breathnach, 1965; Dahm and Landmesser, 1988).

In the immature, developing nerve, a large bundle of axons (greater than 100 axons per bundle) is encompassed by a single layer of Schwann cells. As the Schwann cells proliferate and send their processes deeper into the bundle, the bundle of axons is gradually segregated. The consequence of Schwann cell proliferation and neuronal cell death is a steady decline in the ratio of axons to Schwann cells. One Schwann cell will ensheath several unmyelinated axons. On the other hand, axons destined to be myelinated will continue to be segregated by dividing Schwann cells. Once a 1:1 relationship of Schwann cell-to-axon is achieved, a basement membrane is elaborated from the Schwann cells and PNS myelin formation is ready to commence. As soon as two to six turns of the Schwann cell's plasma membrane are laid down, myelin compaction begins (Peters and Vaughn, 1970; Webster, 1971; Martin and Webster, 1973; Webster et al., 1973).

The length of the myelin internode and the number of spiral wraps increase as the axon grows. The thickness of the myelin sheath in the mature rodent nerve is proportional to axonal caliber. Larger internodes

measuring around a millimeter in length usually contain 50 or more lamellae. If unrolled, the length of the myelin wrap measures roughly two to three times the internodal length (Webster, 1971; Friede and Samorajski, 1967 and 1968; Friede and Bischhausen, 1980). The formation of consecutive internodes along a myelinated fiber is essential to saltatory conduction which has an evolutionary advantage of saving both space and energy during impulse propagation (Ritchie, 1982).

An axon will undergo a process of degeneration, termed Wallerian degeneration, if it is severed from its soma. Schwann cells associated with such degenerating axons tend to dedifferentiate to a pre-myelinating state with proliferating ability. If the axon regenerates, the Schwann cells proliferate and myelination starts again. The myelin sheaths of such regenerated axons are shorter and thinner, consequently, the nerve conduction is slower than normal (Thomas, 1974; Gould, 1985; 1992).

The Components of PNS Myelin

PNS myelin is derived from the spiral wrapping of a Schwann cell process around the axon. The majority of myelin consists of closely packed membrane sheaths. The compaction of the multi-lamellar myelin membrane sheaths may result from its particular composition. With a 80:20 ratio of lipid to protein, myelin has a higher lipid content than other plasma

membranes which have nearly equal amounts of lipid and protein. However, the myelin membrane stacking and close apposition does not appear to depend on the high proportion of the myelin lipid content nor a particular lipid composition (Guidotti, 1972; Kirschner, 1984).

Like other plasma membranes, the major lipid components of myelin are cholesterol, phospholipids, and glycolipids. The molar concentrations of cholesterol, phosphatidylethanolamine, phosphatidylserine plus phosphatidylinositol and sphingomyelin are in the range reported for other plasma membranes with cholesterol as the most abundant species (Kirschner et al., 1984; Norton and Cammer, 1984).

The major proteins in PNS myelin are Po and a number of basic proteins, i.e. the 18.5 kD MBP, the 14 kD MBP, and P2. These proteins together account for about 70% of the total myelin protein (Greenfield et al., 1973, 1980, 1982; Lees and Brostoff, 1984). Many high-molecular-weight proteins, e.g. myelin-associated-glycoprotein (MAG), are present as minor myelin protein components. Another low molecular weight minor component is peripheral myelin protein-22 (PMP-22), a hydrophobic protein with potential growth-regulating ability on Schwann cells (Suter et al., 1992).

Basic proteins account for 18% of the total protein in the PNS myelin and 30% in CNS myelin (Greenfield et al., 1980; Kirschner et al.,

1984; Lees and Brostoff, 1984). The *shiverer* mouse, a mutant without myelin basic proteins (MBPs), exhibit severe myelin deficiency in the CNS, but has near normal myelination in the PNS, indicating that the major basic proteins are essential for CNS myelination but not necessary for normal membrane-membrane interactions in PNS myelin. Since Po protein is not present in CNS and, since it is the only major protein in the PNS of *shiverer* mouse and its cytoplasmic domain is basic like MBP, Po was suggested to account for the formation and maintenance of normal myelin in *shiverer* PNS and presumably also in normal PNS myelin (Kirschner and Ganser, 1980; Kirschner et al., 1984).

The Role of Po in Myelin Compaction

The Structure of Po Protein

Po protein is the most abundant protein in the peripheral nervous system myelin (Ishaque et al., 1980) and its expression is confined to the PNS. It constitutes over 50% of the total PNS myelin protein. Po protein has a molecular weight of 28-30 kD (Greenfield et al., 1973), and is modified post-translationally in several ways (Fig. 2). It is glycosylated (Everly et al. 1973; Roomi et al., 1978) but has a relative low content of carbohydrate of about 6% of its molecular weight (Kitamura et al., 1976; Roomi et al., 1978; Lees and Brostoff, 1984). Po is phosphorylated at serine residues (Brunden and Poduslo, 1987; Suzuki et al., 1990), sulfated in its carbohydrate (Matthieu et al., 1975; Uyemura and Kitamura, 1991) and acylated at Cys153 (Agrawal et al., 1982 and 1983; Sakamoto et al., 1986; Bizzozero et al., 1994).

Po is a single-copy gene. The gene of rat Po is 7 kilobase (kb) long, consisting of six exons (Lemke et al. 1988). The predicted primary sequence of Po protein, based on the analysis of a rat Po cDNA clone (Lemke and Axel, 1985) and also the direct amino acid sequencing of bovine Po (Sakamoto et al., 1987), reveals that the peptide is 220 amino acids (Figure 3). The first 124 N-terminal amino acids represent the

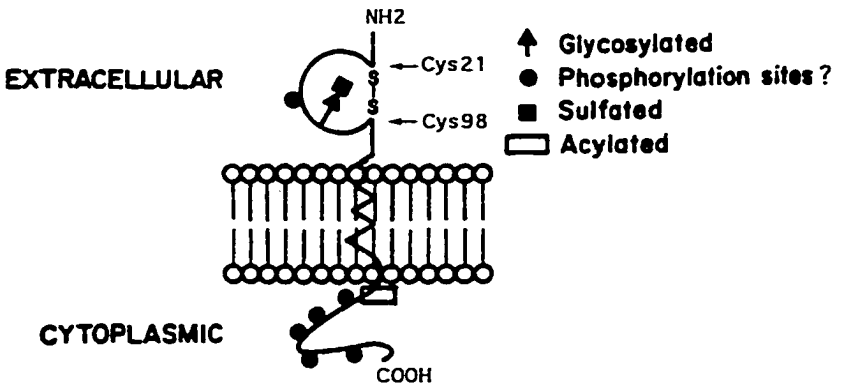


Figure 2: Schematic representation of the location of known and proposed post-translational modifications of Po protein molecule. The circular structure in Po's extracellular domain represents its Ig-like domain. The -S-S- refers to the disulfide bond formed between Cys21 and Cys98. The question mark in the key refers to modifications for which the precise location has to be confirmed.

extracellular domain, followed by a single transmembrane segment of 26 amino acids and a cytoplasmic domain of 69 amino acids. The predicted topology of Po protein molecule was later verified. Using an in vitro translation system, the insertion of Po into rough endoplasmic reticulum (RER) as well as the cleavage of Po's N-terminus were shown to be signal-recognition-particle (SRP)-dependent. This result indicates that the N-terminal region is placed in the luminum of the RER during Po's synthesis, such orientation will lead to the extracellular disposition of Po's N-terminus. Po's membrane topology was further confirmed by means of a cytoplasmic specific monoclonal antibody. This antibody only stained permeablized Po-transfected cells, but not on non-permeablized cells. Taken together with the *in vitro* translational result, Po was therefore

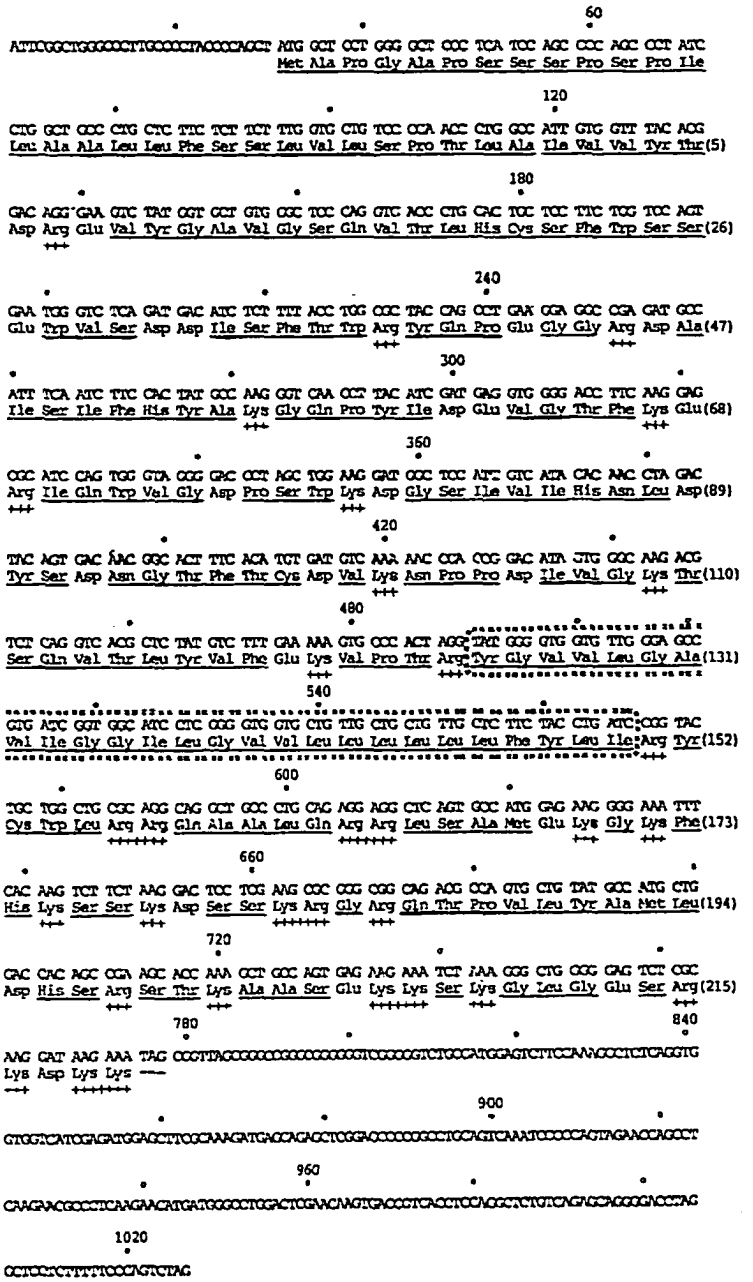


Figure 3. DNA and deduced amino acid sequence of Po molecule. The Po precursor is encoded in nucleotides 32-775. Uncharged amino acids are underlined. +++ denotes basic amino acids. The putative membrane-spanning domain form Tyr125 through Ile150 (nuc. 491-568) is boxed. Numbers in parentheses at the end of each line are amino acids of the mature protein. Reprinted from Lemke and Axel (1985) with permission.

directly shown to be a type I membrane protein with an extracellular N-terminus and a cytoplasmic C-terminal region separated by a single transmembrane domain (Fig. 2 & 6) (D'Urso et al., 1990).

Po's extracellular domain (Po-ED) is N-glycosylated at Asn93 (Lemke and Axel, 1985; Lai et al., 1987). Other features of the Po molecule include a high hydrophobicity in its extracellular segment and high content of basic amino acids in its cytoplasmic domain with 21 basic but only 6 acidic residues (Lemke and Axel, 1985; Lemke, 1988). There are several series of uncharged, hydrophobic residues in Po's extracellular region (e.g., 9-26, 47-54, 110-119) through which hydrophobic bonds could occur (Lemke and Axel, 1985). Based on the predicted secondary structure and primary sequence homology, Po was later included in the immunoglobulin (Ig) gene superfamily (Lai et al., 1987).

The Involvement of Po-ED in Myelin Compaction

Because of its abundance, its hydrophobic extracellular domain and its basic cytoplasmic fragment, and the fact that PNS myelin is virtually unaltered in *shiverer* mouse, Po protein has been suggested to hold together the membranes at both the major dense line and at the intraperiod line (Kirscher and Ganser, 1980; Lemke et al., 1988). In addition, and perhaps unrelated to its adhesive role in myelination, some studies have

also implied a neurite outgrowth promoting function for Po protein (Uyemura et al., 1990; Schneider-Shaulies et al., 1990).

The idea that the Po-ED may mediate adhesion of membrane surfaces is reinforced by the inclusion of Po in the Ig gene superfamily as all members of this family are believed to be involved in recognition/adhesion (Williams and Barclay, 1988; Hunkapillar and Hood, 1989). The *shiverer* mutant mouse lacks MBP, yet has normal PNS myelin repeat periods and separations between the membranes, suggesting that Po is capable of forming and maintaining myelin as it is the only remaining major protein in the mutant mouse (Kirschner and Ganser, 1980; Kirschner et al., 1984). However, it was only later that the homophilic binding capabilities of the extracellular domain of Po was directly demonstrated (Filbin et al., 1990; D'Urso et al., 1990; Schneider-Schaulies et al., 1990; Griffith, 1992).

Po Protein is an Adhesion Molecule

To test the adhesive ability of Po, we expressed an abundance of Po protein in Chinese hamster ovary (CHO) cells by transfection with the Po cDNA (Fig. 4) (Filbin and Tennekoon, 1990; Filbin et al., 1990). In this experiment, a single cell suspension of CHO cells was allowed to adhere. The adhesion was monitored by both periodically counting the total

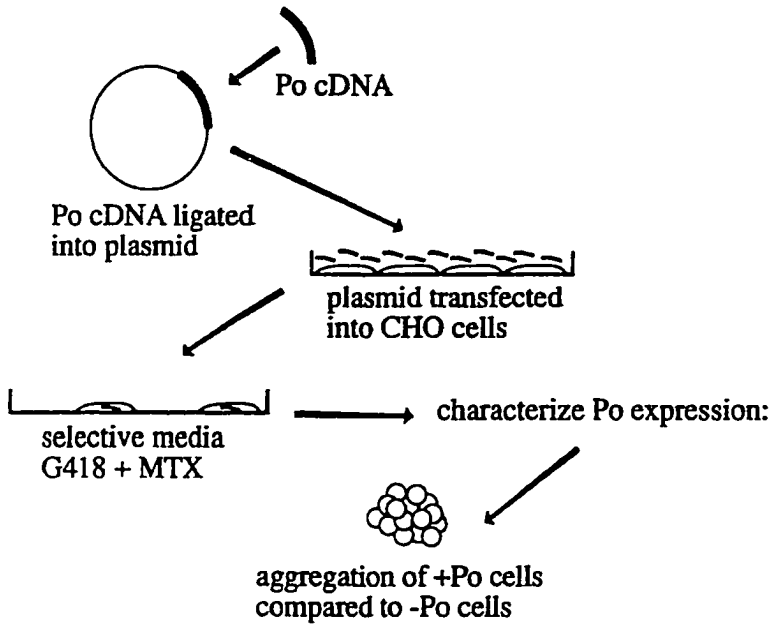


Figure 4: Schematic diagram of procedures to express Po protein in CHO cells. Plasmids containing Po-cDNA were transfected into CHO cells by calcium phosphate precipitation. Transfectants were selected initially in G418 and then for gene amplification by the methotrexate/dihydrofolate reductase strategy. The expression of Po protein was characterized by immunodetection after Western blotting, by its sensitivity to enzyme PNGase F, by immunofluorescent staining on intact cells for surface expression detection, and by ELISA on intact cells for surface expression quantitation. Finally, using adhesion assay, the adhesive properties of the cells expressing Po protein were compared to that of the cells not expressing Po.

particle number (a drop in the number indicates the formation of cell aggregates) and examination of the cell suspension under the microscope for aggregate formation. The cells expressing the Po protein were shown to be at least two orders of magnitude as adhesive as the control transfected cells, i.e. transfected with the vector with the Po cDNA inserted in a 3'-5' orientation. Statistical analysis of data obtained from experiments

mixing Po-expressing cells and control cells showed that Po-expressing and control cells aggregated non-randomly, i.e. Po-expressing cells had adhesive preference for each other rather than for the control cells. Therefore, we also concluded that Po interactions were homophilic.

Furthermore, by expressing a Po cDNA in HeLa cells and using immunocytochemistry with a polyclonal Po antibody, D'Urso et al. (1990) showed that Po protein distributes evenly over the cell surface when cells are not in contact but accumulates at the cell interface when two Po-expressing cells meet. The accumulation of Po protein at the place of membrane contact suggests that Po-ED interacts with Po-ED on the apposing cell membrane. This finding provides further evidence that the extracellular domains of Po adhere homotypically to bring the cell membranes together.

Indeed the involvement of Po in the formation of compact myelin has been demonstrated directly in mice in which no Po is expressed as a result of a null mutation in the Po gene brought about by homologous recombination (Giese et al., 1992). In these transgenic mice, even though compact myelin was found in 10-15% of the PNS nerves examined, the compaction never encompassed the whole myelin sheath. Furthermore, most axons of their peripheral nerves were severely hypomyelinated, many

having loose membrane wrappings but without compact myelin strongly indicating a requirement for Po in the compaction of these membranes.

Possible Roles of Different Components of Po-ED in Adhesion

Studies by us (Filbin and Tennekoon, 1991; Filbin and Tennekoon, 1993) and others (Schneider-Schaules et al., 1990; Griffith, 1992) revealed that both the sugar moieties and the protein backbone of Po are involved in its adhesion. In our studies we showed that the adhesive properties of Po were abolished by either changing the sugar residues from a complex type to a high-mannose type structure (Filbin and Tennekoon, 1991) or eliminating them completely (Filbin and Tennekoon, 1993). We also showed that both molecules in the Po:Po interactive pair must be glycosylated to adhere. However, it is unlikely that the sugar moieties of Po interact directly with each other because, even though a glycosylated Po peptide (amino acid #91-98) was more effective (80% inhibition) in blocking the adhesion of Po-expressing cells, the same peptide unglycosylated was also able to inhibit the adhesion up to 50% (Yazaki et al. 1992). We have found a similar inhibition with both the peptide corresponding to amino acids 91-95 of Po-ED and also with antibodies raised against this peptide sequence (Filbin, 1992; Zhang et al. 1996 and this thesis). In addition, a recombinant form of the unglycosylated Po-ED

was found to bind to itself and to fully glycosylated Po protein (Schneider-Schaules et al., 1990; Griffith et al., 1992). These results indicate that Po is capable of direct protein:protein interaction. We suggest that the role of its sugar residues is to hold its Ig domain in a position away from the membrane from which it extends so that it can interact with the opposing Po molecule.

Po is a Member of the Ig Superfamily

Members of the Ig superfamily have been defined by the presence of one or more regions homologous to the basic structural unit of an Ig molecule, the Ig homology unit or Ig-like domain (Fig. 5) (Hill et al., 1966). The tertiary structure of an immunology unit is referred to as an antibody fold or Ig fold. The primary sequence of the Ig-like domain forms a series of anti-parallel ß-strands which in turn, become the Ig fold of two ß-sheets (Mostov et al., 1984; Williams and Barclay, 1988; Hunkapillar and Hood, 1989).

Since the predicted secondary structure of Po showed the presence of seven ß-strands in its amino-terminal domain, it is included in the Ig superfamily (Lai et al., 1987; Uyemura et al., 1987). The inclusion of Po in the Ig gene superfamily because of the existence of its single Ig-like domain, supports the idea that the Po-ED may mediate adhesion of

membrane surfaces as all members of this family are believed to be involved in recognition/adhesion (Williams and Barclay, 1988; Hunkapillar and Hood, 1989).

Crystal Structure of Po's Extracellular Domain

To determine the crystal structure of Po's extracellular domain, Po-ED was produced as a soluble recombinant protein and allowed to crystallize under conditions near physiological pH and ionic strength (Shapiro et al., 1996). The crystal, examined at 1.9 Å resolution, revealed that Po-ED crystal is made of layers of Po-ED protomers. As predicted from previous amino acid sequence analyses (Lai et al., 1987; Lemke et al., 1988), the crystal structure of Po's extracellular domain resembles the V-like Ig domain (Fig. 30) (Shapiro et al. 1996). Studies on the structure of these layers of protomers suggests Po-ED may project from the membrane as a tetramer (Fig. 31). Each tetramer is linked to four other cyclic tetramers emanating from the opposite membrane leading to a formation of networks of Po-ED (Fig. 32). The C-termini of each tetramer point to the putative membrane surface with the last five residues distorted, suggesting a flexible membrane linkage. In this model, the side chains of Trp28 at the tops of each tetramer would point towards and interact with the opposite membrane. Taken together these results suggest that the homophilic

interaction of Po-ED tetramers at the membrane surface, as well as the direct association of Trp28 with an apposing membrane, cooperate to maintain the exact spacing and adhesion between myelin membranes (Shapiro et al., 1996).

The Role of the Disulfide Bond in the Ig Superfamily

The Structural Characteristics of the Ig Superfamily

The Ig-like domain possessed by the Ig superfamily are characterized by a primary sequence of between 70-110 amino acids in length, usually with a disulfide bond spanning 50-70 residues. One characteristic of the gene structure of the Ig superfamily of molecules is that for the majority of Ig-like molecules the Ig-like domain sequence is usually encoded within one exon (Williams and Barclay, 1988). However, introns were later found to exist between nucleotides coding for the cysteine residues of the conserved disulfide bond in a number of molecules including Po protein (Lemke et al., 1988; Williams and Barclay; 1988). Members of the Ig superfamily carry one or more of the Ig domains. Most of the known polydomains are believed to be derived from gene duplication from one primordial domain (Hunkapiller and Hood, 1989). The primordial gene coding for a domain itself however is proposed to arise from an ancestral half domain structure. Such scenarios for the evolution of the Ig gene superfamily were made based on the observation of the presence of the bisymmetry of homologous sequences in the sequence of an Ig fold (Bourgois, 1975).

The half-domain idea suggests the ancestral half-domain would undergo a dimerization in order to form a functional homodimer unit. A tandem gene duplication could make the ancestral half domain into a single domain. As a result of having a single domain and a 'split' exon of the Ig-like domain, Po protein has been hypothesized to be a close relative to the ancestral gene for the IgG superfamily (Lemke et al., 1988; Williams and Barclay; 1988). Therefore, the adhesive function of Po and the precise mapping of these functional domains may have a much wider implication than simply its role in myelination.

The Subtypes of the Ig Superfamily

There are two types of Ig like domains (Fig. 5), namely variable (V)-like and constant (C)-like folds with similar, but distinctive, tertiary structures. Both V- and C-like domains reveal a sandwich-like structure of 2 ß-sheets that consist of a series of anti-parallel ß-strands, namely strand A, B, C, D, E, F, G. (Willams and Barclay, 1988; Hunkapiller and Hood, 1989). The sequence spanning (about 65-75 amino acids) the two cysteines of the V-like Ig domain is longer than that of the C-like Ig domain (about 55-60 amino acids). As a result, extra ß-strands, the C' and C' strands are formed in the V-like Ig domain (Willams and Barclay, 1988).

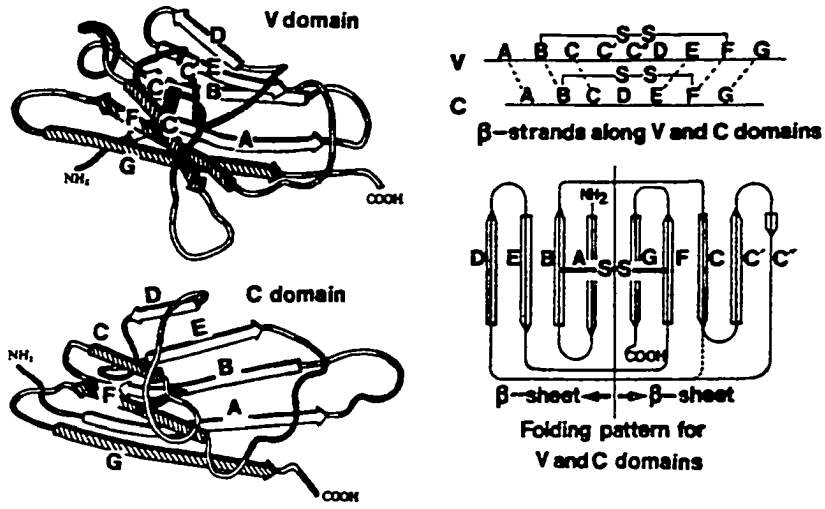


Figure 5: Schematic diagram of Ig-like domains. The folding patterns of both V and C domains are shown. ß-strands are labeled with letters A, B, C, C', C", D, E, F, and G. -S-S- refers to the disulfide bond formed between the B and F ß-strands. In the ribbon diagrams on the left, the shaded ribbons represent one ß-sheet, whereas the un-shaded ribbons constitute another ß-sheet. Reprinted from Willams and Barclay (1988) with permission.

Sequence analysis based on Po cDNA (Lemke and Axel, 1985) demonstrated that the Po-ED contains a pair of cysteines residues (Cys21 and Cys98) separated by 76 residues (Fig. 2 & 3), as well as alternating stretches of ß-strands and ß-turns, thus resembling a V-like domain (Lai et al., 1987; Williams, 1987; Lemke et al., 1988; Saavedra et al., 1989). The V domain is postulated to appear before the C domain in evolution (McLachlan, 1980). As Po carries a V-like domain, this observation fits with the notion of Po possibly being a close relative to the ancestral gene of the Ig superfamily.

Stabilization of the Ig Domain

Most members of the Ig superfamily carry a pair of conserved cysteine residues spaced 55-75 amino acids apart. The 2 ß-sheets of the Ig domain are stabilized by the interaction between the hydrophobic side chains of the antiparallel ß-strands. The structure is believed to be further stabilized by the formation of the disulfide bond between the two conserved cysteine residues to form a compact, globular Ig-like domain that has increased proteolytic resistance (Hunkapiller and Hood, 1989).

The presence of the disulfide bond may not only contribute to the stabilization of Ig-like molecules against enzymatic degradation, a feature postulated for molecules exposed at the cell surface, but may also help maintain and position the active region of the Ig domain in an orientation for adhesion/recognition. However, a correlation between the disulfide bond and the function of the Ig-like domain had never been demonstrated. Since Po carries only one Ig domain which simplifies the interpretation of experimental results, it is an ideal molecule for studying the function of the disulfide bond in homophilic adhesion.

The Role of Po in Guillain-Barré Syndrome

The Characteristics of Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is a demyelinating disease of the PNS. It is considered an immunologically-mediated disease, since an immunological reaction has been shown to be involved in the breakdown of myelin (Ritchie, 1984). Unlike multiple sclerosis (MS), GBS presents as a monophasic disease and is usually preceded by a viral infection. GBS patients develop the symptoms over a range of 1 to 4 weeks, then stabilize and recover over a few weeks to months (Shin and Koski, 1992). The clinical hallmark of GBS is its rapid onset of weakness with only mild sensory involvement (Prineas, 1970). A significant decrease in the maximum conduction velocity correlated with demyelination was often recorded in affected nerves in nerve-conduction studies (Traugott and Raine, 1984).

The Pathogenesis of Guillain-Barré Syndrome

The mechanism of demyelination in GBS is not fully understood. There is an indication that a humoral factor is involved in the decrease of conduction velocity of GBS. It was reported that serum from patients who exhibit GBS produces a conduction block when injected intraneurally into

rat sciatic nerve, indicating that a humoral factor is involved in GBS (Sumner et al, 1982a,b; Ritchie, 1984). In addition, 90 to 95% of the GBS patients were positive for complement-fixing antibodies against peripheral nerve myelin in their serum (Koski et al., 1985, 1986; Shin and Koski, 1992).

Cell-mediated demyelination in GBS has also been shown in cultured tissues of the PNS. Lymphocytes from GBS patients produced demyelination of trigeminal ganglion cultures 48-96 h after their addition (Arnason et al., 1969). To study the mechanism of the autoimmune disease, an animal model for GBS, experimental allergic neuritis (EAN), has been produced (Waksman and Adams., 1955).

The Possible Involvement of Po in Guillain-Barré Syndrome

Experimental allergic neuritis is an inflammatory demyelinating disease of the PNS induced in animals by injection of PNS tissue antigens and complete Freund's adjuvant. During the course of EAN, immune responses against both PNS myelin proteins and lipids have been examined. The proteins studied have included the major PNS myelin specific proteins, Po and P2 (Milner et al., 1987). Although both purified bovine Po and P_2 protein were able to induce EAN in Lewis rats, immune reactivity to P_2 was found to be absent in most GBS patients (Zweiman et

al., 1983; Hughes et al., 1984). Thus Po deserves consideration as an antigen relevant to Guillain-Barré syndrome.

The association between Po and GBS was strengthened by the confirmation of the relation between an outbreak of GBS and the 1976 national immunization program with a swine influenza vaccine. There was a significant increase in the incidence of GBS during the 6 weeks following the swine flu virus vaccine in 1976. Epidemiological analysis on this occasion suggested that the vaccine caused an increased risk of developing GBS in the vaccinated population (Safranek et al., 1991). In an effort to identify the auto-antigen in GBS, comparison between the protein sequences of the influenza virus haemagglutinin and Po shows the presence of a 5 amino acid peptide (SDNGT) shared by both proteins.

In Po, peptide SDNGT is present in the Ig domain of its extracellular domain (Po 91-95). It is possible that the vaccination produced antibodies against the haemagglutinin of the vaccine virus, including one against the SDNGT peptide. Such an antibody may interact not only with the haemagglutinin but also with the 5 amino acids of the Po-ED due to the shared antigenicity. The interaction of the antibody with Po protein is likely to perturb the adhesion between Po protein molecules and cause the myelin sheath to unravel, resulting in PNS demyelination.

The Role of Po in Charcot-Marie-Tooth Disease

The Characteristics of Charcot-Marie-Tooth Disease

Hereditary motor and sensory neuropathies (HMSN) are a heterogeneous group of inherited demyelinating peripheral neuropathies. HMSN include Charcot-Marie-Tooth neuropathy (CMT), Dejerine-Sottas syndrome (DSS), and congenital hypomyelination (CH). These neuropathies are heterogeneous, both pathologically and genetically. There can be clinical and pathological overlap between these demyelinating diseases (Warner et al., 1996). Most cases of CMT show autosomal dominant inheritance.

CMT is characterized by weakness and atrophy, primarily of the peroneal muscles, sensory loss and decreased tendon reflexes, due to slowly progressive segmental demyelination of the peripheral nerves and associated degeneration of axons and anterior horn cells. CMT has traditionally been classified by whether the primary pathological defect is degeneration of the myelin (CMT1) or of the axons (CMT2) in the peripheral nerve. Within CMT1, CMT type 1 A has been mapped to chromosome $17_{p}11.2$, type 1B to chromosome $1_{q}21$-23, type 1C to unknown autosome and X-linked CMT to the proximal $X_{q}13.1$ segment (Bergoffen et al., 1993 a, b). DSS resembles CMT1 clinically and

pathologically, but with a more severe manifestation and earlier onset (Warner et al., 1996).

The Involvement of Po in CMT1B

The Po gene (*MPZ*) has been mapped to the same region as CMT1B, chromosome 1_q22-23 (Hayasaka et al., 1993). To pursue a possible association between CMT1B and the gene of Po protein, the Po gene in all patients of three pedigrees of CMT1B was initially sequenced by three groups of researchers. All CMT1B patients from each of the pedigrees were found to have a point mutation in their Po gene (Hayasaka et al., 1993; Su et al., 1993; Kulkens et al., 1993). Furthermore, all mutations were within the Ig domain of Po-ED which spans Cys21 to Cys98.

In all 3 cases, the point mutations were shown to be genetically linked to the CMT1B locus. Together with the widely accepted view that Po-ED plays an essential role in myelin compaction, Po is suggested to be the gene responsible for CMT1B (Hayasaka et al., 1993; Su et al., 1993; Kulkens et al., 1993). Since then there have been a total of 29 mutations in *MPZ* identified in HMSN patients (Patel and Lupski, 1994; Warner et al., 1996). Among these, 22 mutations were mapped to the same 16 amino acids in Po-ED (Shapiro et al., 1996), whereas 5 mutations affected the intracellular domain of Po (Shapiro et al., 1996; Warner et al., 1996).

In addition, many affected members from CMT1B pedigrees are heterozygous for the normal allele and a mutant allele for the gene of Po protein (Hayasaka et al., 1993; Kulkens et al., 1993; Warner et al., 1996), therefore 50% of the Po protein in CMT1B is normal while the other 50% is aberrant. However, a 50% decrease in gene dosage may not be the only reason responsible for the disease, as heterozygous mice for the Po null mutation have normal myelin until 4 months of age. After 4 months of age, these mice demonstrated a mild form of the disease (Martini et al., 1995).

One alternative mechanism to bring about more severe impairment of the wildtype Po's function is that the aberrant Po accounting for 50% of total Po proteins in these patients, interferes with the functioning of the wildtype Po protein in a dominant-negative manner. Therefore, a dominant-negative effect of the aberrant Po protein upon the normal Po molecule in these patients was proposed (Hayasaka et al., 1993; Kulkens et al., 1993). Studies on the mutations of Po in correlation to the clinical manifestation of HMSN patients lead to the hypothesis that heterozygous loss-of-function mutations reduce the total amount of normal protein, causing a less severe form of CMT1B, while dominant-negative mutations or homozygous mutations in *MPZ* gene produce a more severe form of HMSN, such as DSS and CH (Wong and Filbin, 1996; Warner et al., 1996).

The Cytoplasmic Domain of Po Protein

The Cytoplasmic Domain of Po is Necessary for Cell Adhesion

Po is a transmembrane molecule with a relatively large extracellular domain, a single transmembrane fragment and a relatively small cytoplasmic domain (Lemke and Axel, 1985; D'Urso et al., 1990). A number of *in vitro* and *in vivo* studies have shown that Po behaves like an adhesion molecule holding the extracellular leaflets of the myelin membranes together through the homophilic interaction of its extracellular domains (Kirschner and Ganser, 1980; Filbin et al., 1990; D'Urso et al., 1990; Schneider-Schaulies et al., 1990; Giese et al., 1992). The cytoplasmic region of Po (Fig. 6), on the other hand, is postulated to bring the cytoplasmic sides of the cell membranes together via a heterophilic interaction with acidic lipids in the opposing membrane (Braun, 1984; Lemke et al., 1988). This notion is supported by the finding that the isolated cytoplasmic domain of Po can induce the aggregation of lipid vesicles (Ding and Brunden, 1994).

To determine if the cytoplasmic domain of Po is required for Po adhesion of the extracellular domain, the cDNA's of two truncated Po's with the cytoplasmic domain missing 52 and 59 residues respectively were transfected in to CHO cells (Wong and Filbin, 1994). The adhesive properties of the cells expressing the cytoplasmically-truncated Po's were

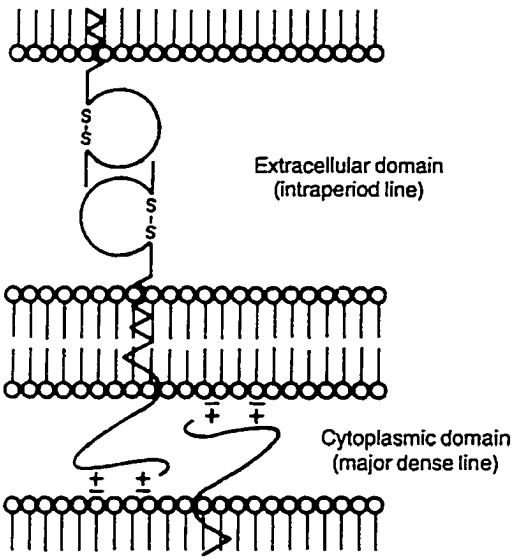


Figure 6: Schematic representation of the proposed interactions of Po's extracellular domain to maintain the intraperiod line of myelin, and of its cytoplasmic domain to form the myelin major dense line. The figure also depicts the orientation of Po protein molecule in the myelin membrane.

assessed with the adhesion assay used to demonstrate the homophilic adhesion mediated by full-length Po (Filbin et al., 1990; D'Urso et al., 1990). Although the truncated Po's were glycosylated and were expressed at the cell surface in amounts similar to the wildtype Po, CHO cells expressing these truncated Po's however were not adhesive. This result suggests that an intact cytoplasmic domain is necessary for the extracellular domain of the Po molecule to adhere homophilically.

In addition, two pedigrees of HMSN have been shown to be related to nonsense mutations in the cytoplasmic domain of Po. These mutations caused a deletion of Po's cytoplasmic domain (69 amino acids) in one of

the families (Nelis et al., 1994), and a truncation of 35 amino acids in another (Warner et al., 1996). The association of the cytoplasmic mutations with the development of neuropathy in these two pedigrees further demonstrates the important role of Po's cytoplasmic sequence in stabilizing the major dense line.

The Interaction of Po's Cytoplasmic Domain with the Cytoskeleton

Although the cytoplasmic domain of Po has been shown to influence the adhesion of Po's extracellular domain (Wong and Filbin, 1994), the mechanism is not known. To study the possible interaction of Po's cytoplasmic domain with the cytoskeleton, CHO cells expressing wildtype and the two truncated Po's missing cytoplasmic 52 and 59 amino acids were treated with the nonionic detergent NP-40 separately. NP-40 will solubilize membrane proteins but not those proteins interacting with the cytoskeleton. Therefore, insolubility in NP-40 is an indication of an interaction of a protein with cytoskeleton.

Extraction of CHO cells expressing the wildtype Po with NP-40 showed that 25-30% of the full length Po was insoluble in the detergent. Treatment of Schwann cells expressing wildtype Po with the same detergent gave a similar insolubility of 25-30% of Po in NP-40, indicating the interaction of Po with cytoskeleton is not exclusive to CHO cells.

However, Po with the cytoplasmic 52 amino acids deleted was far less (only 5-10%) insoluble in NP-40, whereas Po missing the cytoplasmic 59 amino acids was completely soluble in the detergent. These results indicate that deletion of 52 amino acids form the cytoplasmic of Po weakens its interaction with cytoskeleton components, whereas the removal of the additional 7 amino acids from the cytoplasmic domain completely abolishes the interaction of Po with the cytoskeleton (Wong and Filbin, 1994).

The concomitant loss of Po's adhesive property when its cytoplasmic domain is deleted with the loss of its insolubility in NP-40 suggests that the influence of Po's cytoplasmic domain on its extracellular sequence may be mediated by an interaction between the cytoplasmic domain and the cytoskeleton as is the case for some other adhesion molecules such as cadherins (Nagafuchi and Takeichi, 1988; Nagafuchi et al., 1991; Hirano et al., 1992) and integrins (Huang et al., 1993; Gumbiner, 1993). To further test the possibility of the cytoskeleton influencing the adhesion of Po, two cytoskeleton disrupting drugs, cytochalasin and colchicine, were included in the adhesion assay to determine if the adhesive properties of CHO cells expressing the wildtype Po were affected. In the presence of 100 μM colchicine, a microtubule disrupting drug, the adhesion of Po was completely inhibited. In contrast, under the

same conditions, cytochalasin, a microfilament disrupting drug, had no effect on Po's adhesive ability (Wong and Filbin, 1996). Although how the disruption of microtubules affects Po's adhesion requires further study, these results imply a role for the microtubules in the adhesiveness of Po protein.

The Clustering of Po in the Cell Membrane

Clustering of integrin molecules in the same cell membrane has been shown to increase the adhesion to opposing molecules (Hynes, 1992; Gumbiner, 1993). The clustering of integrins is believed to be mediated by an interaction between the cytoplasmic sequence and the cytoskeleton. Integrins truncated in their cytoplasmic domains have been shown to have a dominant-negative effect on wildtype integrins (Balzac et al., 1994; Lukashev et al., 1994; Smilenov et al., 1994). It is possible that Po's adhesion is also mediated by cytoskeleton-dependent clustering in the membrane through the cytoplasmic domain. To test this hypothesis, the cells expressing wildtype Po were treated with a reducible cross-linking agent. After the cells were lysed, Po was immunoprecipitated and separated by running a polyacrylamide gel. Abundance of Po was detected in the gel if Po in the immunoprecipitate was treated with a reducing agent ß-mercaptoethanol immediately before the loading.

However, little or none of the Po was detected if the Po immunoprecipitate was not reduced with ß-mercaptoethanol. The results suggest that when cross-linked, Po molecules in the cell membrane form large aggregates. If a reducing agent is not used before loading the precipitate on the gel, the Po aggregates are too large to enter the 12% gel. Only when the Po aggregates are dissociated with the reducing agent, will Po be able to enter and be detected in the gel. These results in turn suggest the existence of clustering of Po in the cell membrane (Wong and Filbin, 1996).

Dominant-Negative Effect on Adhesion by Po Protein Truncated in Cytoplasmic Domain

To test if the co-expression of each of the truncated forms of Po in the same cell membrane as the wildtype Po affects the adhesion of wildtype Po, Po lacking either the cytoplasmic 52 or 59 amino acids were co-expressed in CHO cells with the wildtype full-length Po. It was found that the truncated forms of Po were fully glycosylated and able to reach to the cell surface in an equivalent amount as the wildtype Po. Although the surface level of wildtype Po in these co-expressors of wildtype and truncated Po's was equivalent to cell lines expressing only wildtype Po, when assessed in the adhesion assay, unlike the cells expressing only wildtype Po the co-expressors were not adhesive. These results suggest

that the co-expression of the truncated forms of Po in the same cell membrane as the wildtype Po abolishes the adhesiveness of the wildtype Po, by a dominant-negative effect (Wong and Filbin, 1996).

To address further this putative dominant-negative mechanism, when the co-expressors, expressing both wildtype and truncated Po, were cross-linked, approximately equal amounts of wildtype and truncated Po were detected after polyacrylamide gel electrophoresis under reducing and non-reducing conditions, suggesting that large aggregates of Po were not formed. This indicates not only that the truncated forms of Po do not cluster in the membrane but that their presence in the same cell membrane as the wildtype, full-length Po prevents the wildtype Po molecules from associating with each other (Wong and Filbin, 1996). The concomitant loss of wildtype Po's adhesiveness with its clustering ability in the presence of the truncated forms of Po, further strengthens the model proposed previously (Wong and Filbin, 1994). This model suggests that not only the adhesion of Po's extracellular domain is influenced by the cytoplasmic sequence, probably through a cytoskeleton-dependent clustering in the membrane, but that a critical number of wildtype Po molecules within a cluster is required for effective adhesion. The co-existence of the non-adhesive truncated Po will dilute the number of the adhesive wildtype Po molecules in each cluster and diminish the adhesion

affinity per cluster to a such an extent, that Po loses the ability to adhere with the opposing membrane (Wong and Filbin, 1996).

Goal of this Thesis Work

The Po protein, the most abundant protein of the PNS has long been proposed to be responsible for holding the membranes of myelin together at both the major dense line, where the cytoplasmic regions of the membranes meet, and at the intraperiod line, where the extracellular surfaces come together. In line with the hypothesized role of Po protein in PNS myelin compaction, we (Filbin and Tennekoon, 1990; Filbin et al., 1990) and others (D'Urso et al., 1990; Schneider-Schaulies et al., 1990; Griffith et al., 1992) have shown that Po is capable of behaving like a homophilic adhesion molecule. However, the mechanism of Po adhesion remains largely unknown.

In addition, Po has been shown to be mutated in some cases of HMSN (Hayasaka et al., 1993; Su et al., 1993; Kulkens et al., 1993; Warner et al., 1996), a group of hereditary demyelinating diseases of the PNS, which further implicates an important role of Po during PNS myelination. Nevertheless, direct testing of Po's involvement in the disease as well as the pathogenic mechanism of the disease needs to be carried out.

Hence, the objectives of this thesis were to (1) map the functional regions of the Po protein within the Ig domain; (2) address the role of the disulfide bond in Po's Ig like domain; (3) determine if there is a dominant-negative effect of a mutated Po protein on Po's adhesive function.

Aim #1: What Amino Acid Sequences in Po's Ig-Like Domain Are Critical for Its Adhesion?

In order to map the functional regions of the Ig-like domain in the Po-ED, peptides corresponding to three regions, Po amino acids 38-46, 74-82 and 91-95, of the Ig-like domain of Po-ED will be synthesized and used to raise antibodies in rabbits. The ability of these antibodies, as well as their corresponding peptides, to inhibit Po-Po interaction will be assessed. In addition, among the 5 amino acids of the Po peptide 91-95 (SDNGT), amino acid Asp92 and Gly94 are highly conserved in a subset of Ig-like molecules (Williams and Barclay, 1988) and as they are evolutionally conserved, are likely to be functionally important to these Ig like molecules. Hence, the role of Asp92 and Gly94 in adhesion will be studied by replacing both residues with a conservative amino acid respectively, and the effect of such mutation on Po's adhesion of Po will be assessed.

Aim #2: Does the Disulfide Bond of Po Play a Role in its Homophilic Adhesion?

To assess the role of the disulfide bond in the adhesion of Po its formation will be prevented from forming by altering the codons of one of the 2 cysteines, Cys21 at one end of Po's Ig-like loop. The mutated Po cDNA will then be transfected into CHO cells, and the effect of this

mutation on adhesion will be assessed and compared to the CHO cells expressing the wildtype Po.

Aim #3: Does Mutated Po Protein have a Dominant-Negative Effect on Wildtype Po's Adhesive Function?

Many individuals suffering from CMT1B are heterozygous for the mutant and the normal alleles of the Po gene, hence, half of the Po produced by these individuals is normal. It is possible that the aberrant CMT1B Po protein (from the mutant allele) interferes with the normal Po molecule (from the wildtype allele) and prevents it from functioning through a dominant-negative effect. This would result in dysmyelination. Such a possibility will be tested here, by introducing the Cys21-mutated Po (from Aim #2), an unadhesive protein, into a well-characterized CHO cell line already expressing the wildtype Po protein and shown to be adhesive. The effect of the mutated protein on the ability of the wildtype Po to adhere will then be addressed.

Chapter II

Mapping the Adhesive Domains of Po

Introduction

As it is the most abundant protein in the myelin of the peripheral nervous system (PNS) (Ishaque et al., 1980), Po has long been proposed to be responsible for holding together the myelin membranes at both the major dense line, via its cytoplasmic domain, and at the intraperiod line, via its extracellular domain (Kirschner and Ganser, 1980; Braun, 1984; Lemke and Axel, 1985). Indeed, we (Filbin et al., 1990) and others (D'Urso et al., 1990; Schneider-Schaulies et al., 1990), have shown that Po protein does behave like an adhesion molecule. However, the detailed mechanism of Po:Po interaction at the molecular level remains to be determined.

As it contains a V-like Ig domain, Po is included in the Ig superfamily. Because of the simplicity of its structure and its 'split' exon coding of the Ig domain, Po has been proposed to be the closest molecule to the ancestral gene of the Ig superfamily (Barclay et al., 1987; Williams and Barclay, 1988). Therefore, structure-function studies with Po's Ig domain may be pertinent to the Ig superfamily.

The initial studies addressing the mechanism of Po-Po interaction revealed that both carbohydrate (Filbin and Tennekoon, 1991) and protein backbone (Schneider-Schaulies et al., 1990; Griffith et al., 1992) are involved in the homophilic adhesion of Po protein. However, a

combination of results from us and others indicated that it is unlikely that the sugar component interacts directly in the homophilic adhesion of Po protein molecules. Rather, we suggested that Po's carbohydrates may hold the peptide of its Ig domain in an optimal conformation for the homophilic adhesion (Filbin, 1992; Schneider-Schaules et al., 1990; Griffith et al., 1992).

To identify the functional region of Po protein's Ig like domain, antibodies were raised against three peptides within Po's Ig domain, Po 38-46, 74-82, 91-95. These three antibodies will be tested for their effect on Po's adhesion. The choice of the three peptides was based on their high hydrophilicity from a hydrophobicity plot, as hydrophilic residues are more likely to be exposed on a folded molecule's surface and are therefore in a position to interact with another Po molecule. Not only will the three antibodies be assessed for their inhibitory effect on Po's adhesion, to exclude the possibility of steric hindrance by these three antibodies, the three peptides themselves will also be tested in an adhesion assay for the same assessment. Furthermore, preliminary results showed that of the three antibodies, the antibody against Po peptide SDNGT (Po 91-95) was the most effective in inhibiting Po's adhesion. Therefore, Po 91-95 (SDNGT) will be studied in more detail. We plan to study Po peptide SDNGT by mutating two amino acids, the residue 92 and 94. Residues Asp92 and

Gly94 were chosen because these two amino acids are highly conserved in the Ig superfamily, hence, they may be functionally important.

To study whether residues Asp92 and Gly94 are directly involved in Po's adhesion, residue 92 will be mutated from Asp (D) to Glu (E), and residue 94 will be altered from Gly (G) to Ala (A), both conservative changes. A conservative replacement of amino acids is employed here to avoid the loss of function caused by a disruptive structural change. The cDNAs of the Po mutated at residues 92 and 94 (D92E/G94A Po) will be transfected into CHO cells to test the effect of the mutations on Po adhesion, using the adhesion assay.

Materials and Methods

Cell Culture:

Dihydrofolate reductase (dhfr) mutant CHO cells (Urlaub and Chasin, 1980) were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FCS and proline (40 mg/liter) at 37°C in 5% CO_2. For untransfected cells, thymidine (0.73 mg/liter), glycine (7.5 mg/liter), and hypoxanthine (4.1 mg/liter) were added. For transfected cells, DMEM with 10% dialyzed FCS (dDMEM) was used, hypoxanthine was omitted, and 100 nM $CdCl_2$ was added.

Preparation of Peptide Antibodies:

After commercial synthesis, Po peptides 38-46, 74-82 and 91-95 were coupled to an antigen carrier, the keyhole limpet hemocyninn (KLH) by means of their SH groups from the cysteine that was additionally incorporated at their N-termini. The peptide-KLH conjugates, emulsified with complete Freund's adjuvant, were then used to immunize rabbits. Rabbits were bled 10 days after the injection and after centrifugation, the serum was obtained. Boosts were carried out 4 and 8 weeks after the initial immunization.

Sera collected before and after immunization were tested for their ability to recognize the entire Po protein molecule on a western blot of total cell lysate of Po-expressing CHO cells. Although the affinities varied, all the peptide antibodies recognized the intact Po molecule, denatured by SDS on a western blot. Furthermore, indirect immunofluorescence of live, Po-expressing CHO cells showed that all these antibodies were able to recognize native Po protein. Thus, antibodies against all three peptides from the Ig domain of Po molecule were ready to be tested for their ability to block the adhesion of Po.

Peptide Synthesis:

Peptides used for the adhesion inhibition assay correspond to the amino acid sequences 38-46 (RYQPEGGRD), 74-82 (GDPSWKDGS), 91-95 (SDNGT) of Po's Ig like domain. After being commercially synthesized on HMP resin, the peptides were dissolved in 70% acetonitrile/H_2O/0.1% trifluoroacetic acid and purified by reverse-phase HPLC with a C18 column.

Adhesion Assay:

Cells at 80-90% confluence were washed with PBS and incubated with 5U/ml trypsin (GIBCO) for 2-3 min at room temperature, washed

twice with dDMEM, and finally resuspended in dDMEM containing 10% fetal calf serum by 4 passages through an 18-gauge syringe. Suspensions, containing a minimum of 95% single cells, were diluted to a final concentration of 1.5-2 X 10^6 cells per ml and allowed to aggregate at 37 °C in 5% CO_2 with gentle rocking at 5 rpm.

Before sampling, the tubes were gently inverted twice and aliquots were removed at intervals, examined under the microscope, and their total particle number was determined with a Coulter counter. Where indicated cells were preincubated for 1h at 37 °C while rocking at 30 rpm to prevent aggregation, with either antibody at a concentration of 100 μg/ml or with 0.5-20 mM peptide.

To allow adhesion to occur, cells were incubated at 37°C while rocking at 5 rpm. At least three separate incubations were performed for each experiment, and duplicate samples were withdrawn at each time point and counted three times each. Specific adhesion was calculated as the % drop in total particle number of the CHO cells expressing wildtype Po minus the % drop in total particle number of the control transfected CHO cells, not expressing Po.

Mutation of Po cDNA at Residues Asp92 and Gly94

The nucleotides coding for Asp92 and Gly94 in Po amino acids #91-95 (SDNGT), were altered to Glu92 and Ala94 respectively, using a polymerase chain reaction (PCR) strategy. Briefly, 2 oligonucleotides corresponding to sequences 176 basepairs (bp) apart on the cDNA of Po were used as primers for the PCR reactions. Each primer contained a unique restriction enzyme site to facilitate the subcloning of the amplified sequence later.

The 3' primer annealed to the sequence corresponding to the Asp92 and Gly94 region, contained a substituted codon for Glu in place of the original Asp at residue 92 (a conservative substitution) and a substituted codon for Ala instead of for the original Gly at residue 94 (also a conservative substitution). Using the 1 kilobase (kb) wildtype Po cDNA as the template, the amplified products were obtained with replaced nucleotides coding for both residues #92 and #94.

Ligation of Po cDNA Mutated at Asp92 and Gly94 into a Suitable Plasmid

Using the unique restriction enzyme site at either end of the primers, Cla I for the 3' primer, Aat II for the 5' primer, the amplified mutated Po cDNAs were used to replace the corresponding sequence on the wildtype

Po cDNA carried in pbluescript (pBS). Mutations were confirmed by di-deoxy sequencing (DNA sequencing kit from US Biochemicals). The 1 kb fragment of the mutated Po cDNA was then subcloned into pSJL (Fig. 7), a eukaryotic expression vector, at a unique *Xho* I cloning site downstream

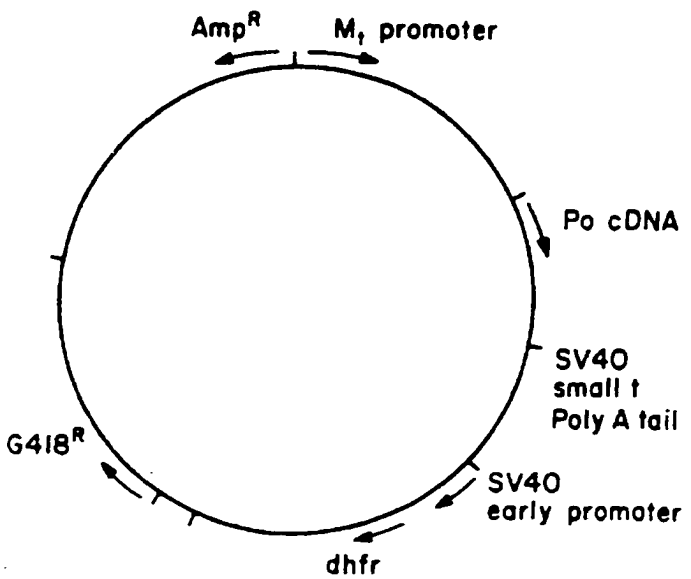


Figure 7: Diagram of the plasmid SJL (pSJL) containing Po-cDNA used to transfect and express Po protein in the CHO cells. Po cDNAs were subcloned into pSJL at a unique *Xho* I restriction enzyme site downstream from a metallothionein promoter and upstream from a poly (A) tail of the SV40 small t antigen gene. pSJL contains a G418 resistant gene as a selection marker in transfected cells, and a dihydrofolate reductase (*dhfr*) gene for gene amplification. pSJL also carries an ampicillin resistance gene for growth and selection in *E. Coli.*

from the mouse metallothionein promoter and upstream from the poly (A) tail of the SV40 t-antigen gene (Lee and Nathans, 1988; Filbin and Tennekoon, 1990). The plasmid also contained the genes for G418 resistance and dihydrofolate reductase (*dhfr*). The orientation of the Po

cDNA in the plasmid was confirmed by restriction enzyme digestion. The pSJL plasmid containing the mutated Po cDNA was then transfected into Chinese hamster ovary (CHO) cells.

Transfection:

The dihydrofolate reductase (DHFR) mutant Chinese hamster ovary (CHO) cell line was transfected with 1 μg DNA per 6-cm plate, by calcium phosphate precipitation (Graham and van der Eb, 1973) followed by a glycerol shock (Frost and Willams, 1978). The cells were passed, 1:2, the following day, and 3 days after transfection. 400μg/ml of active G418 was added to the culture medium. Colonies usually appeared after about 3 weeks and a number were picked, expanded, screened for the expression of Po by Western blot analysis. Several of these single-cell clones were used for gene amplification.

Gene Amplification:

Cells with multiple copies of the *dhfr* gene were selected by growing the cells in increasing concentrations (0.05-10 μM) of methotrexate (MTX). Cells were plated at 5 X 10^5 cells per 10 cm plate, and those surviving after 2-3 weeks at each concentration of MTX were allowed to multiply before being replated on the higher concentration of

MTX. At each stage in the gene amplification procedure, cells were monitored for Po expression by Western blot analysis.

Immunodetection of Po Immobilized on PVDF Membrane:

Cell (80-90% confluent) were lysed in 0.5M Tris-HCl, pH 7.2, containing 2% sodium dodecyl sulfate (SDS) and 4% ß-mercaptoethanol and the following antiproteases: leupeptin 1µg/ml; antipain, 2µg/ml; benzamidine, 10µg/ml; chymostatin, 1µg/ml; pepstatin, 1µg/ml; and phenylmethylsulfonylfluoride, 1µg/ml. The lysate was homogenized by passage through a tuberculin syringe (protein was measured with a Bio-Rad kit), and incubated at 95°C for 3 min and subjected to SDS-PAGE on a 12% gel (Laemmli, 1970). The proteins were then transferred to PVDF membrane and immunostained (Filbin and Poduslo, 1986) overnight at 4°C with anti-bovine Po serum at a dilution of 1:3000. Secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (1:15000) (Sigma). 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) was used as a substrate and Nitro Blue Tetrazolium (NBT) as a chromogen according to the manufacturer's instructions (K.P.L.).

PNGase F Treatment of Po Protein:

PNGase F is also known as peptide-N-glycosidase F, peptide-N-(acetyl-ß-glucosaminyl) asparagine amidase, and N-glycosidase F, recombinant. It cleaves all types of asparagine bound N-glycans provided that the amino-group as well as the carboxylgroup are present in a peptide linkage and that the oligosaccharide has the minimum length of the chitobiose core unit. The reaction products are ammonia, aspartic acid (in the peptide chain) and the complete oligosaccharide. The reaction mechanism differs from that of endoglycosidases D, H and F. These enzymes cleave the glycosidic linkage between the two N-acetylglucosamine residues. They also show a more limited substrate specificity than PNGase F. The extent and rate of deglycosylation of glycoproteins depend to a high degree on the nature of the glycoprotein. Therefore, there are no general instructions with regard to the incubation conditions. According to the instruction sheet provided by Boehringer Mannheim, PNGase F deglycosylates a number of glycoproteins in their native form, but denaturation (e.g. by heating at 100°C in the presence of SDS) increases the deglycosylation rate considerably. After SDS denaturation it is necessary to add a second detergent (e.g. N-octylglucoside, Triton X-100, Nonidet P-40) to the denatured sample before adding PNGase F in order to avoid inactivation of the enzyme by

SDS. This detergent should be present in a 5-10 fold excess compared to the concentration of SDS.

Cells, 80%-90% confluent, were lysed in 50mM Tris-HCl (pH8.6) containing 25mM EDTA, 0.1%SDS, 0.5% NP-40, 1% ß-mercaptoethanol in a volume of 500 µl/plate. The lysate was then boiled for 3 minutes and allowed to cool to room temperature. Following this, 2 µg/ml antipain, 1 µg/ml leupeptin, 10 µg/ml benzamidine, 1µg/ml chymotrypsin, 1µg/ml pepstatin and 0.1mM phenylmethylsufonyl fluoride were added. The mixture from each plate was divided into two tubes: one was control without PNGase F and to the other one 2 ul of PNGase F (1000 U/µl New England Biolab) and 1/10 volume 10X reaction buffer provided by the company were added. All tubes were incubated at 37°C for 1 hour. Proteins in each tube were precipitated with 8 volumes of acetone at -20°C overnight and brought down by being centrifuged at 3000 rpm for 20 min. Each pellet was allowed to dry and resuspended in 200µl of sample buffer containing 0.1% glycerol, 2% SDS, and 0.03M pH 6.8 Tris and subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained for Po as described above.

Quantitation of Po Expressed at the Cell Surface:

The ELISA was carried out as previously described by Doherty et al. (1990), modified as follows. Between 2000 and 3000 cells per well were plated in a 96-well ELISA plate and allowed to attach and grow for 2 days. The cells were rinsed twice with PBS and fixed for 30 min with 4% paraformaldehyde. The cells were then rinsed with PBS. Nonspecific binding sites were blocked for 30 min with 3% normal goat serum in DMEM, and the cells were incubated overnight at 4°C with rabbit anti-bovine Po serum (1:1500) in DMEM containing 1% normal goat serum. The cells were rinsed, nonspecific binding sites were again be blocked for 30 min with 3% normal goat serum in DMEM, and cells were incubated for 1 hr with horseradish peroxidase(HRP)-conjugated goat anti-rabbit IgG (1:1500) (Sigma). The cells were rinsed with PBS and then with water. Color was developed by the addition 50 µl of 0.2% (wt/vol) o-phenylenediamine (OPD) (Sigma) and 0.02% (vol/vol) H_2O_2 in citrate buffer (pH 5.0) to each well. The reaction was stopped after 15 min by the addition of 50 µl of 4.5 M H_2SO_4 and the optical density at 490 nm was determined by using a 96-well plate reader. All incubations were at room temperature unless stated otherwise. Controls consisted of control transfected cells incubated with anti-bovine Po antibody and Po-expressing cells with no primary antibody. For each experiment, 80 wells

were assayed for each cell line at least three times. The average number of cells per well was estimated by counting cells using a Coulter counter after their removal with trypsin, from 5 separate wells for each 96-well plate. Results were standardized to absorbance unit per cell.

Detection of Po at the Cell Surface by Indirect Immunofluorescence:

Cells were grown on 8-well tissue culture slides (Lab-Tek). Cells were fixed with 4% paraformaldehyde for 15 min and then washed with DMEM. The cells were then blocked for 30 min at room temperature with DMEM containing 10% FBS and then incubated with rabbit anti-bovine Po polyclonal antibody (1:50) overnight at 4 °C. After washing with DMEM and again blocking, the cells were then incubated with anti-rabbit biotinylated antibody (1:300) for 30 min at room temperature. After washing, the cells were incubated with streptavidin conjugated Texas red (1:300) for 30 min at room temperature. The cells were then washed, post-fixed and mounted with Gel-mount and viewed with a Zeiss fluorescence microscope.

Results

Characterization of Po-peptide Antibodies

The choice of Po-peptides for antibody production was based on a hydrophobicity plot of its extracellular sequences. Sequences of hydrophilic amino acids were chosen because they are more likely to be exposed at the surface of the molecule and hence in a position to interact with an opposing Po molecule. Based on this analysis three peptide sequences were chosen: Po 38-46, Po 74-82 and Po 91-95. Polyclonal antibodies were raised to these peptides, and the antibodies were characterized as previously described in Materials and Methods; all of the peptide antibodies recognized Po on a western blot and on the surface of intact cells.

The Effect of Po-peptide Antibodies on Po:Po Adhesion

We demonstrated previously the ability of Po to behave as a homophilic adhesion molecule via interactions of its extracellular domain (Filbin et al 1990). This was carried out through an aggregation/adhesion assay using transfected CHO cells expressing Po protein. Briefly, in this assay a single-cell suspension was incubated and aggregation was monitored both by microscopic examination and by counting the total

particle number at various times. This permits both a qualitative (microscopic examination) and quantitative (counting) analysis since a decrease in total particle number correlates with aggregate formation, and the rate of aggregate formation is an indication of the strength of adhesion.

To determine if the Po-peptide antibodies could inhibit Po:Po interaction, an Ig-fraction prepared from each antiserum was included in the aggregation/adhesion assay. Each Ig-fraction (100 μg/ml) was preincubated with the Po-expressing cells before aggregation was permitted to proceed (rocking the cells at 30 rpm prevents aggregation). After 60 min incubation under aggregating conditions (37°C; rotating at 5 rpm) only antibodies to Po 91-95 inhibited adhesion completely (Fig. 8). Antibodies to Po 74-82 inhibited adhesion by about 20% above the control, while antibodies to Po 38-46, and to Po 190-210 in the cytoplasmic domain, had no effect on adhesion (Fig. 8). When examined under the microscope, by 60 min, the Po-expressing cells, in the absence of antibody, had formed large aggregates as we previously reported (Filbin et al., 1990; Filbin and Tennekoon 1991, 1993). Similar large aggregates were observed when cells were incubated with antibodies to either Po 38-46 or Po 190-210. In contrast, when peptide antibody Po 91-95 was included in the assay, no large aggregates were observed; the aggregation was indistinguishable from that of control transfected cells (results not shown).

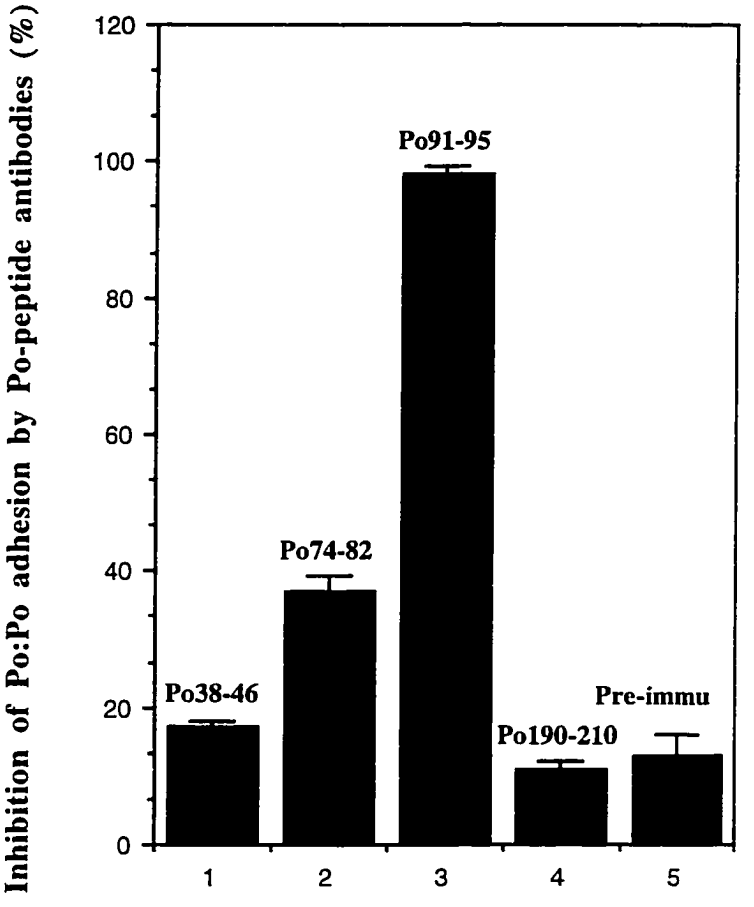


Figure 8: Inhibition of Po:Po-mediated adhesion by Po-peptide antibodies. Cells expressing Po protein were pre-incubated for 60 min, with antibodies against peptides (100 μg/ml) corresponding to amino acids Po 38-46 (**column 1**), Po 74-82 (**column 2**), Po 91-95 (**column 3**), Po 190-210 (**column 4**), or pre-immune serum (**column 5**) before commencing the adhesion assay. After 60 min incubation under aggregating conditions, samples were withdrawn and the total particle number counted. 100% specific adhesion was calculated as the drop in total particle number of the Po-expressing cells minus the drop in total particle number of the control cells, not expressing Po. Results represent at least three experiments, each in triplicate, +/- the SE.

Furthermore, antibodies to Po 91-95 had no effect on the non-specific adhesion of the control transfected cells. Likewise, an Ig-fraction prepared

from preimmune serum had no effect on the adhesion of Po-expressing cells (Fig. 8).

The Effect of Po-peptides on Po:Po Adhesion

The ability of Po 91-95 antibodies to inhibit adhesion indicates that Po 91-95 may be directly involved in Po's adhesion. However, the antibody can inhibit the adhesion either because its epitope is the adhesive domain that is directly involved in the interaction or prevent adhesion because of steric hindrance. To complement the results of antibody inhibition and exclude the possibility of a steric hindrance effect, the peptides themselves were included in the adhesion assay. The peptides were incubated with the single cell suspension at a concentration of 0.5-20 mM, at 37°C for 30 min while rotating at 30 rpm prior to the start of the adhesion assay.

It was found that, like its corresponding antibody, peptide SDNGT inhibited the adhesion of Po-expressing CHO cells. The inhibition of adhesion by SDNGT was shown to increase as its concentration was increased, reaching 100% inhibition at approximately 3mM (Fig. 9). This result strengthens the suggestion that the sequence SDNGT is important in the adhesion of Po. Although significantly less effective than peptide SDNGT, the peptide 74-82 also inhibited Po:Po adhesion; at a

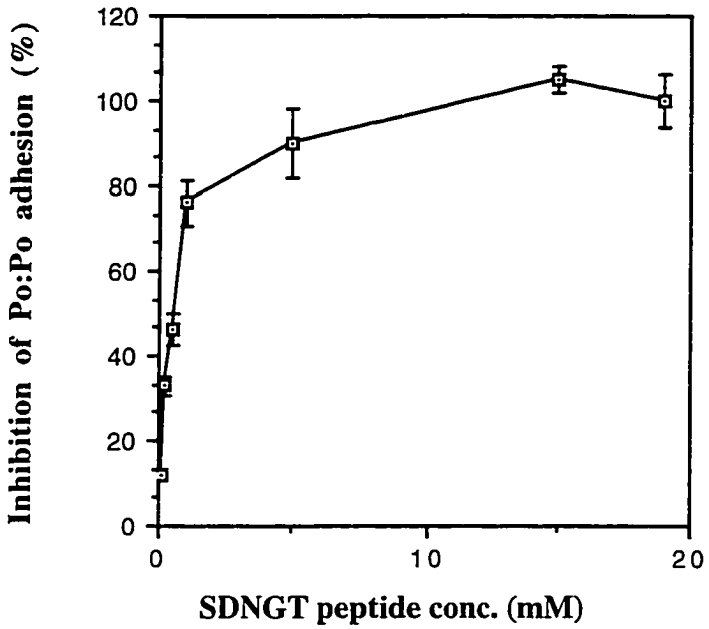


Figure 9: Dose dependence of inhibition of Po:Po-mediated adhesion by Po-peptide 91-95, SDNGT. Cells expressing Po protein were pre-incubated with various concentrations of Po 91-95 before commencing the adhesion assay. After 60 min samples were withdrawn and the total particle number counted. 100% specific adhesion was taken as the drop in total particle number of the Po-expressing cells minus the drop in total particle number of the control cells, not expressing Po. Results represent at least three experiments, each in triplicate, +/- the SE.

concentration of 16mM, Po 74-82 inhibited adhesion by approximately 80%. In contrast, peptide 38-46 inhibited adhesion by only 12%, which is no different from that with the control peptide (Fig. 10).

Expression of D92E/G94A Po in CHO Cells

To determine if the two residues in peptide SDNGT, Asp92 and Gly94 are critical to Po's adhesive function, the codons for the two amino

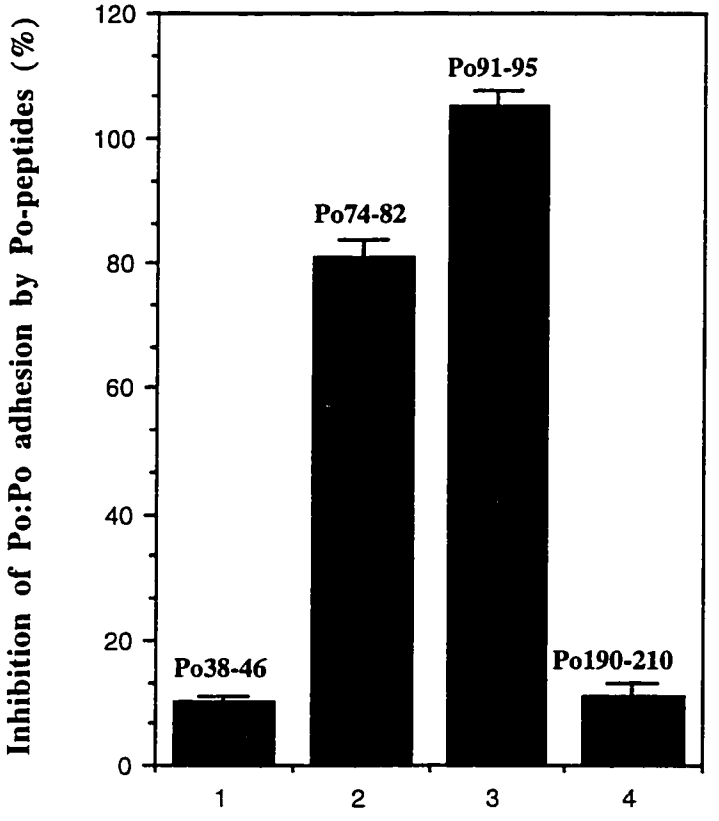


Figure 10: Inhibition of Po:Po-mediated adhesion by Po-peptides. Cells expressing Po protein were pre-incubated for 60 min, with Po peptides (16 mM) corresponding to amino acids Po 38-46 (**column 1**), Po 74-82 (**column 2**), Po 91-95 (**column 3**), Po 190-210 (**column 4**) before commencing the adhesion assay. After 60 min incubation under aggregating conditions, samples were withdrawn and the total particle number counted. 100% specific adhesion was calculated as the drop in total particle number of the Po-expressing cells minus the drop in total particle number of the control cells, not expressing Po. Results represent at least three experiments, each in triplicate, +/- the SE.

acids were mutated to Glu and Ala respectively. The mutated Po was termed D92E/G94A Po. The mutated Po cDNA in vector pSJL was transfected into CHO cells. Transfectants were selected in G418 and the expression of a few clones was amplified with methotrexate (MTX). The

mutated D92E/G94A Po-expressing cells were characterized and compared to CHO cells expressing the wildtype Po.

A Western blot of the cell lysates from these clones was used to monitor the expression level of the mutated Po protein. Figure 11 shows the result from one of these clones. On this Western blot, each lane contains 30 μg of protein. The level of expression of the mutated Po protein was equivalent to that of the clone expressing the wildtype Po. In addition, by treatment with PNGase F, the mutated Po was shown to be glycosylated to the same extent as wildtype Po. The apparent molecular weight of the mutated Po was decreased to the same extent as the wildtype Po after the removal of the sugar, i.e. by approximately 6%.

The Po mutated at Asp92 and Gly94, like the wildtype, was shown to reach to the cell surface (Fig. 12). This was carried out by indirect immunofluorescence of fixed, unpermeablized cells using anti-Po antibody. Both mutated and wildtype Po-expressing cells stained for Po at the cells' surface, while the control cells, not expressing Po protein, gave only background staining (Fig. 12).

Before the adhesive properties of the clone expressing the mutated Po were compared to the cells expressing wildtype Po, the surface expression level of the mutated Po protein was measured by ELISA on fixed, unpermeablized cells with anti-Po antibody (Fig. 13). The results

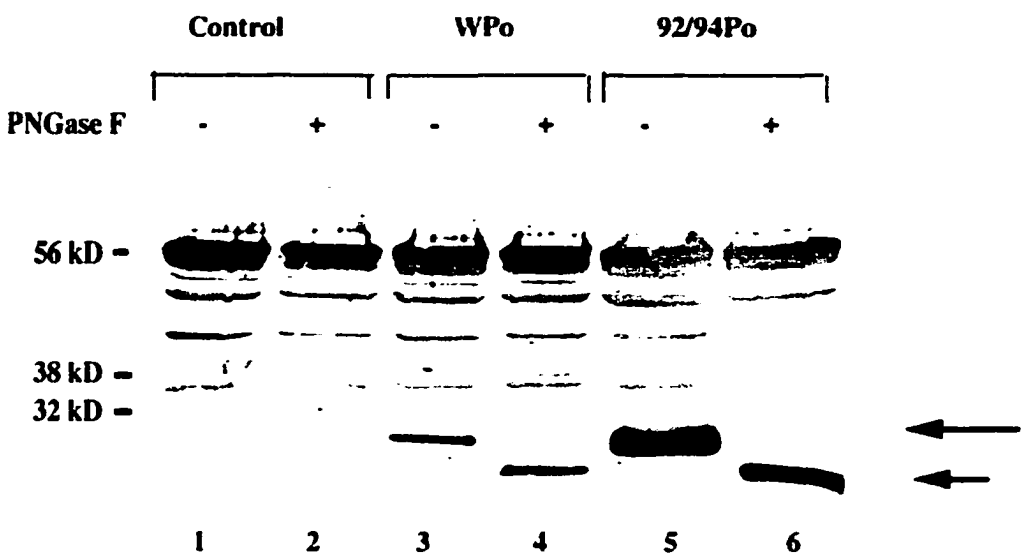


Figure 11: Effect of PNGase F treatment of Po mutated at Asp92 and Gly94, expressed by transfected CHO cells. Proteins (30 μg/lane) from cell lysates with (+) and without (-) treatment with PNGase F, Control transfected **(lanes 1 and 2)**, transfected with the wildtype Po cDNA **(lanes 3 and 4)** or with the Po cDNA mutated at Asp92 and Gly94 **(lanes 5 and 6)** were separated by SDS-PAGE and immunostained for Po. Long arrow refers to glycosylated Po; short arrow, deglycosylated Po. Bars indicate molecular weight standards from top to bottom as follows: 56, 38 and 32kD.

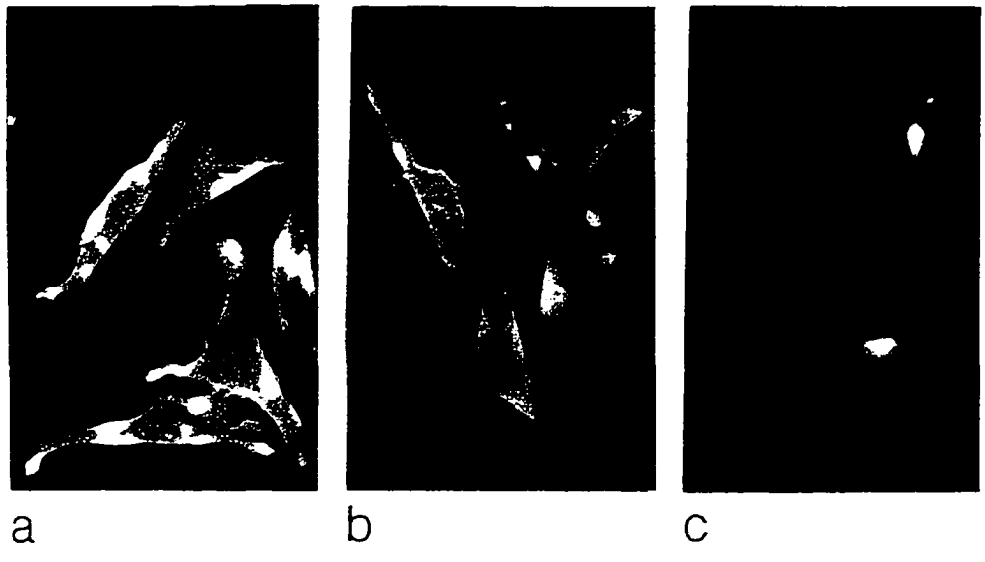


Figure 12: Detection of Po on intact cell surface by indirect immunofluorescence: (**a**) cells expressing wildtype Po; (**b**) cells expressing Po mutated at Asp92 and Gly94, and (**c**) control transfected cells.

show that the mutated Po reaches the surface in an equivalent level as the wildtype Po protein, while the control-transfected cells gave only a background signal.

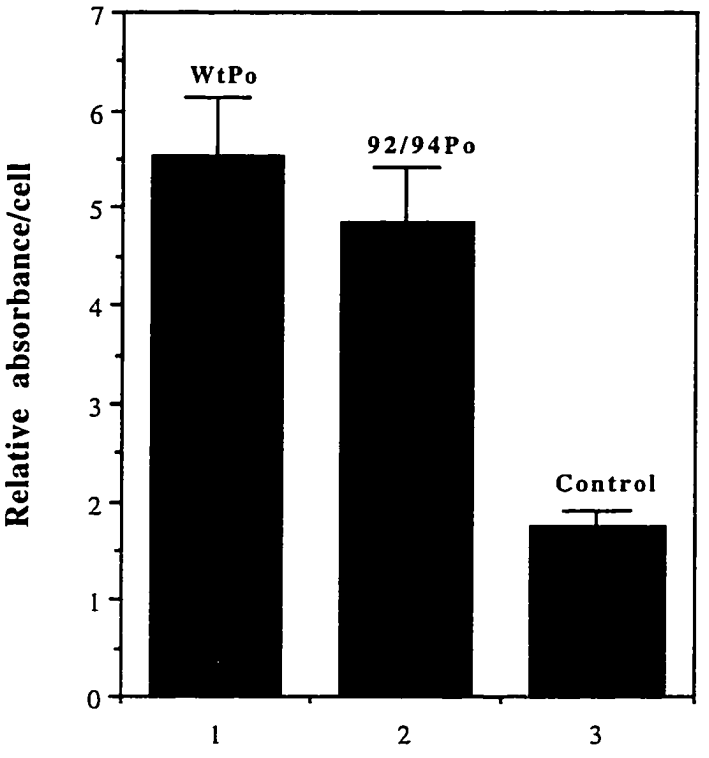


Figure 13: Quantitation of Po mutated at Asp92 and Gly94, expressed at the transfected cell surface. The relative amount of Po expressed at the cell surface was quantitated by an ELISA for transfected cells expressing wildtype Po (**column 1**, WtPo), Po mutated at Asp92 and Gly94 (**column 2**, 92/94 Po) and control-transfected cells (**column 3**, control), using a Po-peptide antibody directed against sequences in the extracellular domain. Results are expressed (+/- SE) in relative absorbance units per cell and are the mean of three experiments, 40 samples per experiment.

Adhesion of Cells Expressing D92E/G94A Po Protein

Since the clone expressing the mutated Po was shown to express nearly the same level of Po protein at the surface as the CHO cells

expressing the wildtype Po, this clone was assessed for its homophilic adhesiveness by the adhesion assay. By 120 minutes, the cells expressing the wildtype Po had formed large aggregates, as previously described (Filbin et al., 1990; Zhang and Filbin, 1994) (Fig. 14). In addition, the total particle number of the cells expressing wildtype Po had dropped to about 20% of the starting number (Fig. 15), another indication that adhesion had occurred, consistent with the microscopic examination. After the same time, the cells expressing the mutated Po protein remained mostly as single-cells, doublets or triplets, and were indistinguishable from the control cells, not expressing Po. The total particle number of the cells expressing the mutated Po dropped to about 60%, as did the control cells. These results demonstrate that the cells expressing Po mutated at 92 and 94, are not adhesive.

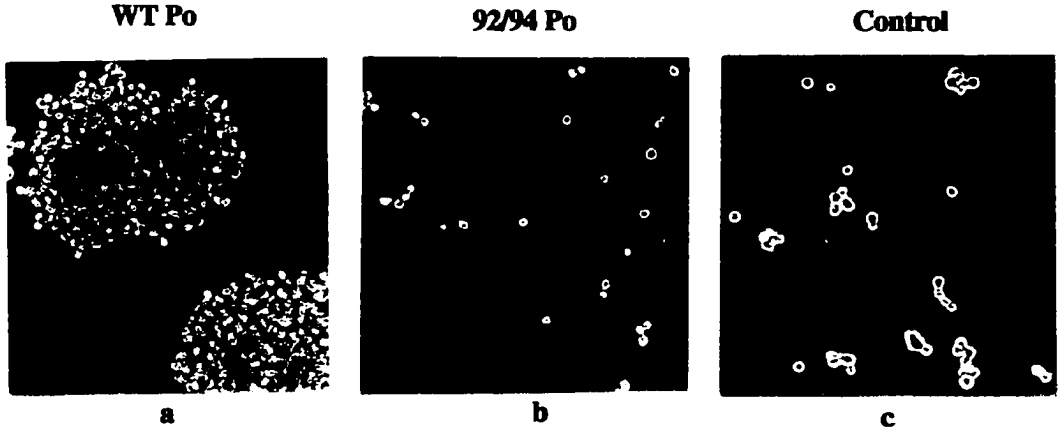


Figure 14: Aggregation properties of cells expressing Po mutated at Asp92 and Gly94, examined under microscope. Single-cell suspensions of CHO cells expressing (**a**) wildtype Po, (**b**) Po mutated at Asp92 and Gly94, or (**c**) control-transfected cells not expressing Po, were allowed to aggregate. Samples were withdrawn at intervals and examined under the microscope (results after 120 min aggregation).

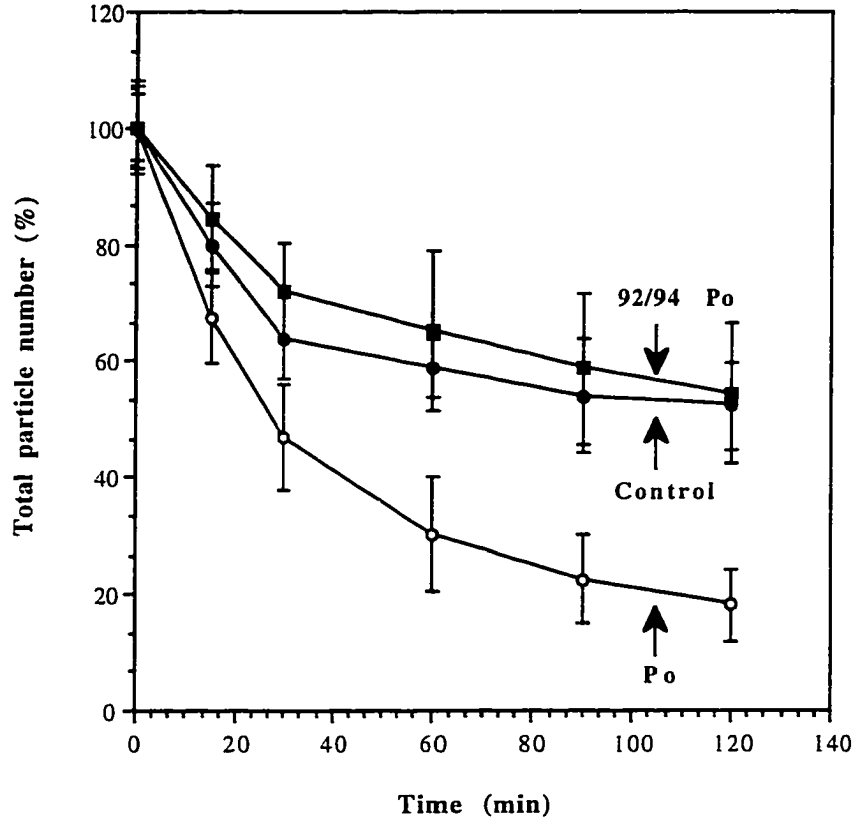


Figure 15: Aggregation properties of cells expressing Po mutated at Asp92 and Gly94, characterized by adhesion assay with Coulter counter. Single-cell suspensions of CHO cells expressing wildtype Po, control-transfected cells, or Po mutated at Asp92 and Gly94, were allowed to aggregate. Samples were withdrawn at intervals and examined under the microscope (Fig. 14) and the total particle number was counted in a Coulter counter. The total particle number +/- SE was plotted against time.

Summary

1. After 60 min incubation in the adhesion assay, antibodies against peptide 91-95 and 74-82 inhibited the adhesion of Po-expressing CHO cells by 100% and 40% respectively. The antibody against peptide 38-46 inhibited adhesion by only 20% which was indistinguishable from the control cells. These results suggest that Po peptide 91-95 may be directly involved in Po's adhesion and peptide 74-82 may be partially involved. The peptide 38-46 probably does not participate in adhesion.

2. The adhesion assay with the three peptides demonstrated that peptide 91-95 (SDNGT) inhibited the aggregation of CHO cells expressing wildtype Po. Inhibition increased as the concentration of SDNGT peptide increased. The inhibition reached 100% at about 3 mM SDNGT. In addition at a concentration of 16 mM, the peptide 74-82 inhibited adhesion by about 70%, while peptide 38-46 inhibited by only about 12%, indistinguishable from the control cells. These results agree with the results from the study with the peptide-antibodies, strengthening a direct involvement of peptide SDNGT and a partial involvement of peptide 74-82 in the homophilic adhesion of Po. Peptide 38-46 is probably not involved directly in adhesion.

3. The adhesion assay with Po expressing cells showed that by 120 minutes, the cell expressing the wildtype Po formed large aggregates and, the total particle number dropped to about 20% of the starting number. In the same time, cells expressing the Po mutated at Asp92 and Gly94 did not form large aggregates and the total particle number dropped to only about 60% of the original reading, indistinguishable from the control cells not expressing Po. This indicates that the mutated Po was not capable of homophilic adhesion.

Chapter III

Po's Disulfide Bond

Introduction

The primary sequence of an Ig domain is about 70-110 amino acids in length (Hunkapiller and Hood, 1989). The majority of Ig domains contain two cysteines, with one near either end of the domain. In the few Ig domains that do not have the two cysteines, a hydrophobic amino acid often replaces the cysteine. These hydrophobic residues are thought to stabilize the Ig domain by their in-pointing sidechains (Williams and Barclay, 1988).

The Ig domain can be aligned into a series of anti-parallel ß-strands of 5-10 residues and subsequently into 2 ß-sheets (Fig. 5). The tertiary sandwich-like structure of the ß-sheets is believed to be mediated by interactions between the hydrophobic side chains of the ß-strands and also by the formation of a disulfide bond between the two cysteines (Hunkapiller and Hood, 1989). Therefore, the conserved disulfide bond may be important to the structure and, consequently, the adhesion/recognition function of Ig-like domains.

As Po contains only one Ig domain, it is an ideal molecule with which to address the relationship between the conserved disulfide bond and the tertiary structure, as well as the adhesive function of an Ig molecule. To address this question, the disulfide bond of Po was

prevented from forming by replacing one of the two cysteines, Cys21 with alanine through site-directed mutagenesis on Po's cDNA. Then, the Po cDNA devoid of Cys21 was transfected into CHO cells. After the expression of the mutated Po was characterized, the CHO cells expressing the Po devoid of its disulfide bond were assessed for their adhesive capabilities.

Materials and Methods

Mutation of Po Residue #21 from Cysteine to Alanine

2 oligonucleotides corresponding to sequences 153 basepairs (bp) apart on the Po cDNA were used as primers for the PCR reaction (Fig. 16). While both primers contained a unique restriction enzyme site (BstE II and Cla I respectively), one of them superimposed to the segment of the sequence where the Cys21 was. This primer was made to contain a codon of alanine at residue #21 instead of the cysteine codon. Therefore, by using the 1 kilobase wildtype Po cDNA as a template, the 153 bp long amplified product from these 2 primers contained an alanine residue at amino acid #21 instead of the usual cysteine.

Ligation of Po cDNA Mutated at Cys21 into a Suitable Plasmid and Transfection into CHO Cells

Through the unique restriction sites of the primers, the Cys21-mutated, 153 bp long DNA product was used to replace the corresponding sequence in the wildtype Po cDNA carried on plasmid pBS. After DNA sequencing with a sequenase kit (US Biochemicals) to ensure the presence of the mutation (Fig. 17), the Cys21- mutated Po cDNA was ligated into a

eukaryotic expression vector pSJL (Fig. 7) and transfected into CHO cells (Fig. 4) (see Chapter II Materials and Methods).

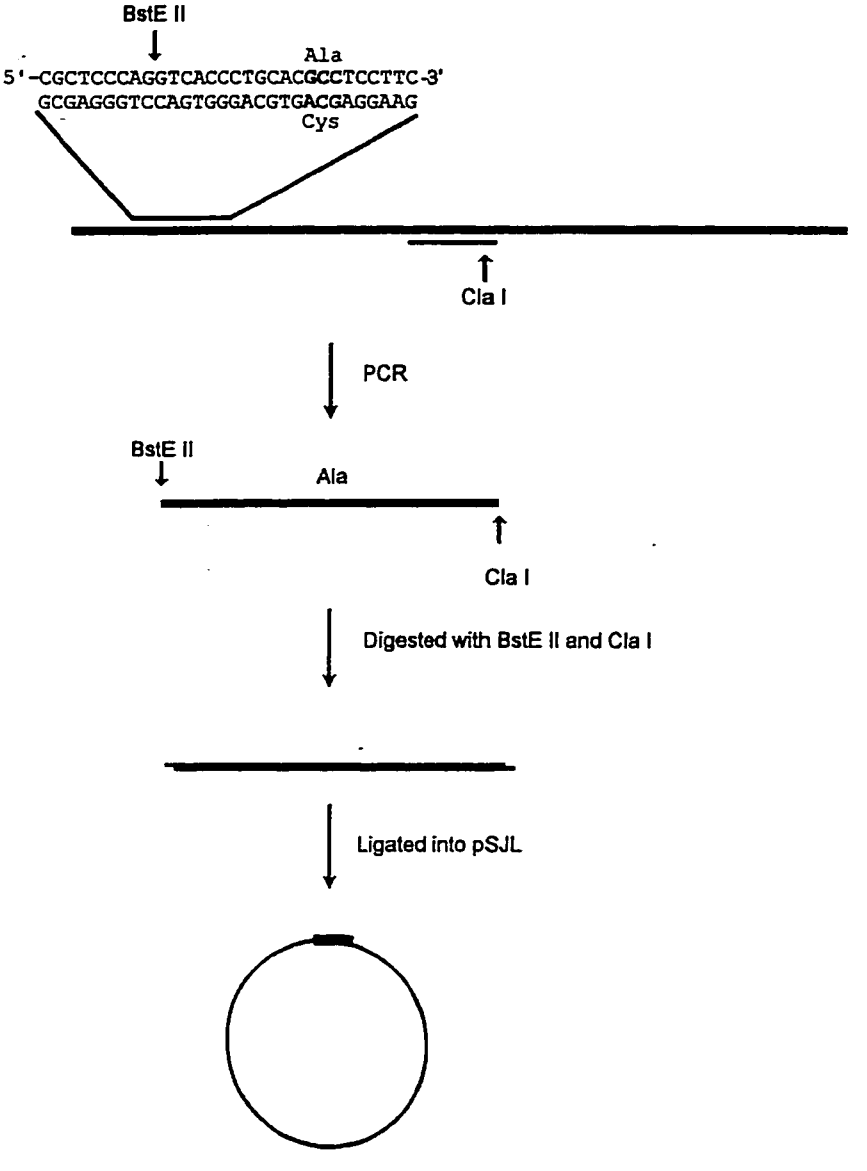


Figure 16: Schematic diagram of procedures to mutate Po residue #21 from cysteine to alanine using PCR technique. Two oligonucleotides corresponding to sequences 153 basepairs apart on Po-cDNA were used as primers. Each primer contained the sequence coding for a unique restriction enzyme site (BstE II and Cla I respectively) and one also contained the mutated codon (Ala) for amino acid #21. The 1 kilobase unaltered Po-cDNA was used as the template for the PCR. The 153 basepair amplified product was purified and ligated onto a eukaryotic expression vector, pSJL.

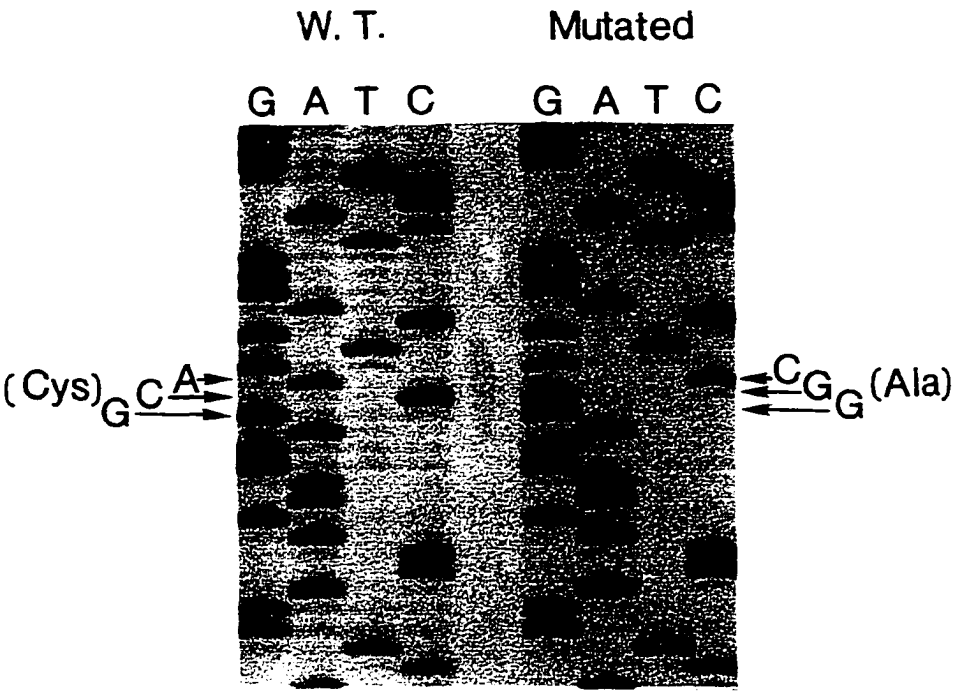


Figure 17: Autoradiograph of sequencing gel showing the mutation in Po residue #21 from nucleotides coding for cysteine to nucleotides coding for alanine. Nucleotides TGC code for cysteine, GCC is one of the codons for alanine.

Gene Amplification

Initially, the positive transfectants were selected by growing the transfected CHO cells in 400 μg/ml of G418. 18 individual clones obtained from G418 selection were screened for their Po protein expression by western analysis using rabbit anti-bovine Po serum. Then the Po expression level of one such clone was increased by using the dihydrofolate reductase gene amplification strategy (Filbin and Tennekoon, 1990). The cells were grown in sequentially increasing concentrations (0.05-3 μM) of methotrexate (MTX) to increase their expression of the Cys21-mutated Po protein.

Characterization of Expression of Mutated Po by CHO Cells

The mutated Po expressed by this clone was characterized for its molecular weight and glycosylation by Western blot and PNGase F treatment (see Chapter II Materials and Methods). The existence of the surface expression and the level of such surface expression were demonstrated by ELISA and indirect immunofluorescence. Finally the adhesive ability of the Cys21 mutated Po was assessed by adhesion assay (see Chapter II Materials and Method).

Results

Expression of Cys21-Mutated Po in CHO Cells

To determine if the disulfide bond is essential for Po protein to adhere, we replaced Cys21 with alanine (C21A Po), a conservative substitution to prevent Po's disulfide bond from forming. After mutating the nucleotide coding for Cys21 via a PCR strategy, the mutated cDNA was transfected into CHO cells and expression of the mutated Po protein was amplified by the dhfr/MTX strategy. The level of expression of the mutated Po protein during gene amplification was monitored by Western blotting and staining with anti-bovine Po antibody. Following this, we characterized the mutated Po protein expressed by one of the clones.

First, the apparent molecular weight of the Cys21-mutated Po protein was examined by Western blot using the total cell lysate and was found to be the same size as the wildtype Po which is about 30 kD under reducing conditions (Fig. 18). Wildtype Po protein's apparent molecular weight on a polyacrylamide gel is 28 kD under non-reducing condition, and 30 kD under reducing condition (Cammer, 1980). However, Cys21-mutated Po did not show a shift in its molecular weight when proteins were separated with and without ß-mercaptoethanol (Fig. 18, lane3 and 4). This indicates that the mutated Po was indeed devoid of a disulfide bond.

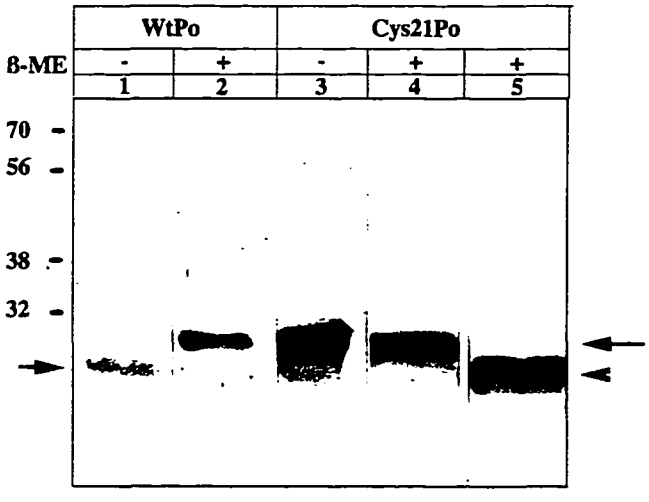


Figure 18: Western blot immunostained for Po of cells expressing wildtype Po protein (**lanes 1 and 2**) or Cys21-mutated Po (**lanes 3-5**): **lanes 1 and 3**, without ß-mercaptoethanol; **lanes 2,4 and 5**, with ß-mercaptoethanol; **lane 5** treated with PNGase F. For each lane 100 μg of protein was used. Arrowhead, deglycosylated Cys21-mutated Po; short arrow, non-reduced wildtype Po; long arrow, reduced wildtype Po, reduced and non-reduced Cys21-mutated Po. Molecular weight standards are, from top to bottom, as follows: 70, 56, 38, and 32 kD.

With PNGase F treatment, the extent of C21A Po's glycosylation was also shown to be the same as the wildtype Po. After the carbohydrates of the Cys21-mutated Po were removed by treatment with PNGase F, the apparent molecular weight decreased by about 6%. This demonstrates that Cys21-mutated Po is glycosylated to the same extent as the wildtype Po (Fig. 18, lane 5).

Fig. 18 shows that the level of expression of Po per 100 μg of total protein for the Cys21-mutated or the wildtype Po in the two cell lines is comparable. To confirm that Po was reaching the cell surface in the two cell lines, immunofluorescence of intact cells was carried out. Figure 19 shows that using indirect immunofluorescence staining of fixed, unpermeabilized transfected cells, the intensity of staining was comparable for both cell lines. This indicates that the mutated Po, like the wildtype Po, reaches the cell plasma membrane (Fig. 19) which suggests that these two cell lines are appropriate for comparing their adhesive properties.

However, before the adhesive properties of the cells expressing the mutated Po can be compared with the wildtype Po, surface expression of the two cell lines must be compared. Quantitation of surface expression was carried out using an ELISA on fixed, unpermeabilized cells. The mutated Po-expressing cells were shown to express equivalent amounts of Po on the surface as the cells expressing the wildtype Po (Fig. 20).

Figure 19: Surface indirect immunofluorescent staining for Po of fixed, unpermeabilized CHO cells expressing (**a**) wildtype Po, (**b**) Cys21-mutated Po, and (**c**) control transfected cells.

Therefore, these cells were suitable for the adhesion assay with the mutate Po.

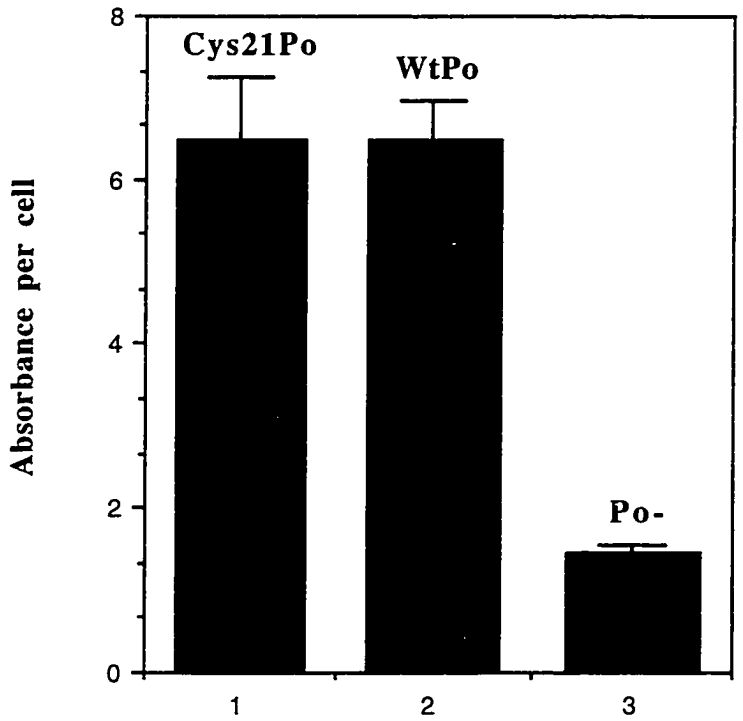


Figure 20: Quantitation by ELISA of Cys21-mutated Po expressed at the surface of transfected CHO cells. An ELISA was carried out on fixed, unpermeabilized cells. **Column 1**, cells expressing Cys21-mutated Po; **2**, cells expressing wildtype Po; **3**, control transfected cells. The Po protein mutated at Cys21 is reaching the cell surface in amount equivalent to the unaltered protein.

Adhesion of Cys21-Mutated Po

To assess any change in the adhesive function of the mutated Po protein, the adhesion assay was carried out as previously described (Filbin et al., 1990). First, a single cell suspension was each prepared for cells expressing the mutated Po and for cells expressing wildtype Po.

Aggregation was allowed to proceed at 37 °C while rotating the cells at 5 rpm. Samples were withdrawn at intervals and examined for aggregate formation by microscopic observation and counted for total particle number.

As we reported previously (Filbin et al., 1990), by 60 min the cells expressing the wildtype Po had formed large aggregates and the total cell particle number had dropped to approximately 20% of its starting value. In contrast, the total particle number of cells expressing the Cys21-mutated Po had barely changed, and was indistinguishable from the control cells not expressing Po protein. Likewise, at this time, microscopic examination revealed no aggregate formation of the cells expressing the mutated Po or the control cells not expressing Po (Fig. 21). This indicates that, unlike the wildtype protein, a Po devoid of its disulfide bond does not behave like a homophilic adhesion molecule.

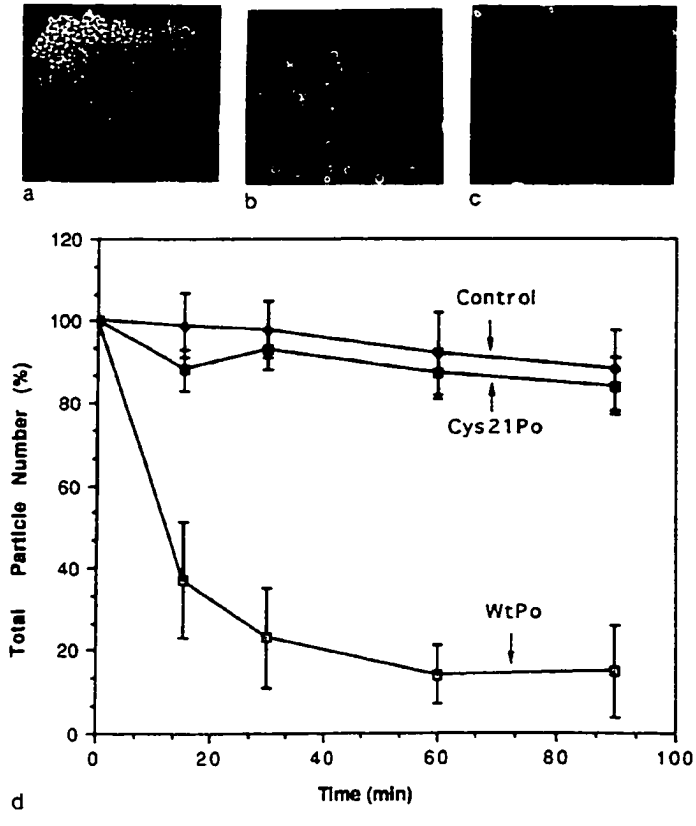


Figure 21: Aggregation properties of cells expressing Cys21-mutated Po. A single cell suspension of cells expressing (**a**) wildtype Po, (**b**) Cys21-mutated Po, or (**c**) control transfected cells was allowed to aggregate. Samples were withdrawn at various intervals and examined under the microscope (**a-c** represent results after 60 min aggregation) and counted in a Coulter counter for total particle number. **d:** Total particle number versus time. Data are mean +/- SE (bars) values of 3 experiments.

Summary

1. The Cys21-mutated Po protein has the same apparent molecular weight as the wildtype Po protein.

2. As expected, unlike the wildtype Po, the Cys21-mutated Po did not show a shift in its apparent molecular weight under reducing and non-reducing conditions.

3. The Cys21-mutated Po protein is glycosylated to the same extent as the wildtype Po protein.

4. The Cys21-mutated Po protein was able to reach to the cell surface in levels comparable to the surface expression of the wildtype Po.

5. Cells expressing the Cys21 mutated Po protein were not able to adhere homophilically.

Chapter IV

The Effect of Mutated Po on the Adhesion of Wildtype Po

Introduction

Initially, three studies on pedigrees of CMT1B co-localized the Po gene with the CMT1B locus (Hayasaka et al., 1993; Kulkens et al., 1993; Su et al., 1993). To search for the corresponding gene for the disease, the Po gene in all the affected individuals of the 3 pedigrees was sequenced and shown to carry mutations in the Po gene. Hence, the mutations in Po were suggested to be responsible for CMT1B. Since then, 29 point mutations in the human Po gene (*MPZ*) have been identified in patients with hereditary motor and sensory neuropathies (HMSN) including CMT1B (Warner et al., 1996), further confirming the causative role of Po mutations in this heterogeneous group of diseases.

One of the features of CMT1B is that most of the affected individuals are heterozygous for the mutant allele of the Po gene. Consequently, the level of wildtype Po protein in these patients will be half of that of the normal individuals. Hence, not enough wildtype Po protein molecules could be one of the reasons for the disease. Nevertheless, this may not be enough to account for the whole spectrum of severity of HMSN. In addition, heterozygous mice for the 'knocked-out' Po gene, which also have 1/2 the normal Po gene dosage, appeared to have

normal myelination up until 4 months of age, after which they developed a mild form of demyelination (Martini et al., 1995).

A second possibility is that the mutated Po proteins in HMSN patients are capable of preventing the wildtype Po molecule from functioning normally, namely they produce a dominant-negative effect. Such a scenario would in turn require that in order to interact with the apposing molecule efficiently, Po may need to associate with other Po molecules on the same cell membrane, i.e. Po may oligomerize.

In CMT1B there may be two different Po molecules or monomers. One is the normal monomer resulting from the normal allele of the Po gene, the other is the mutant monomer from the mutant allele. Based on the hypothesis of possible dominant-negative effect of the mutated Po, and assuming all mutant-monomer-containing oligomers are not functional, the number of oligomers containing only wildtype monomers in CMT1B will dramatically decrease. This in turn could lead to the manifestation of the disease.

Whether Po monomer mutated in the extracellular domain, can interfere with the wildtype Po monomer and prevent it from functioning can be assessed by the transfection/adhesion assay system we have established to demonstrate the homophilic adhesion function of Po (Filbin et al., 1990). Initially, the CHO cell line expressing the wildtype Po, and

shown to be adhesive, will be re-transfected with the Cys21-mutated Po cDNA. In this way, cells expressing both wildtype and Cys21-mutated Po proteins, co-expressors, will be obtained. The adhesive properties of such co-expressors will then be assessed and compared to cells expressing only the wildtype Po.

Although none of the mutations identified in CMT1B occur at Cys21, Cys21-mutated Po will be used here because it is known to be a surface expressing, nonfunctional protein (see Chapter III). It is unlikely that all the 29 mutations identified so far in the MPZ , contribute directly to the adhesive domain of Po. Instead, some of the mutations, as with Cys21-mutated Po, are likely to cause a loss of function by disrupting the tertiary structure of the Po molecule. Therefore, Cys21-mutated Po may mimic the behavior of some of the mutations in CMT1B and serve as a good model to test if mutated Po can have dominant-negative influence on its wildtype counterpart.

Materials and Methods

Subcloning the Cys21-Mutated Po onto pCEP4

The 1 kb Cys21-mutated Po cDNA (see Chapter III Materials and Methods) was sub-cloned into a eukaryotic expression vector, pCEP4 (Invitrogen) (Fig. 22), at a unique *Xho* I restriction enzyme site. pCEP4 carried a hygromycin resistant gene as a selectable marker rather than the G418 resistant gene, as the cells were already G418 resistant.

Transfection of Cys21-Mutated Po into CHO Cells Expressing Wildtype Po

Using calcium phosphate precipitation and a glycerol shock (see Chapter II Materials and Methods), the pCEP4 carrying the Cys21-mutated Po cDNA, was transfected into CHO cells already expressing the wildtype Po protein and shown to be adhesive (Fig. 23). After selection in 250 U/ml of hygromycin, 18 colonies were picked and screened for the presence of both the wildtype and the mutated-Po protein. These two types of proteins are known to be distinguished by their behaviors in the presence and absence of 2% ß-mercaptoethanol.

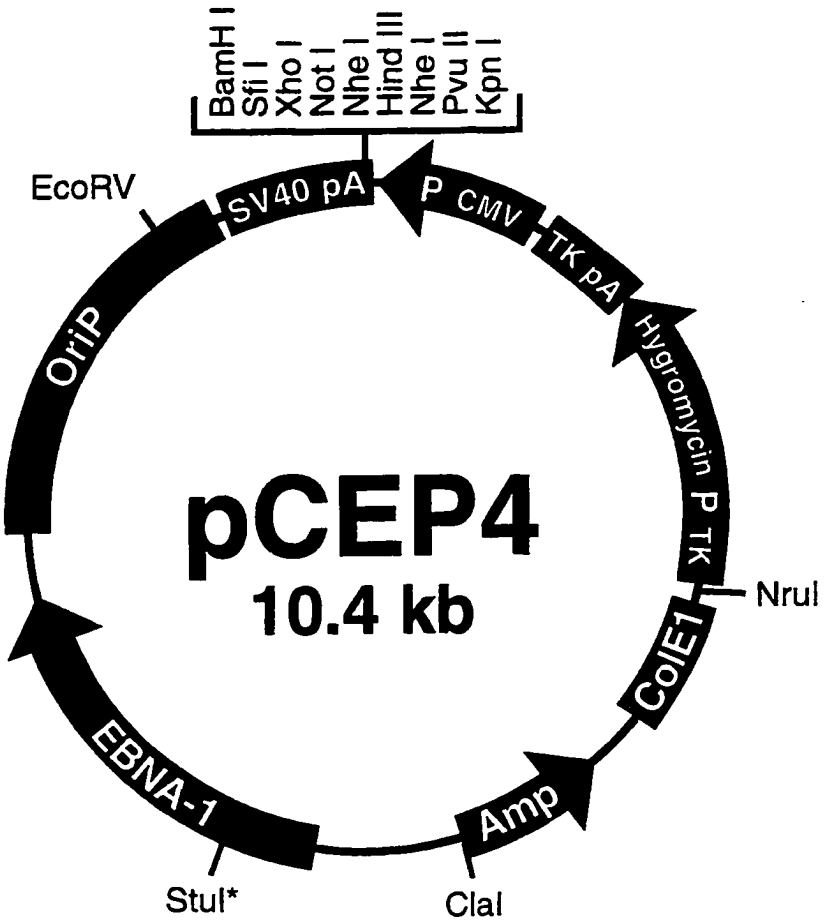


Figure 22: Diagram of plasmid pCEP4 used to subclone the Cys21-mutated Po and then to co-transfect and co-express the mutated Po with the wildtype Po protein in the CHO cells. The Cys21-mutated Po cDNA was subcloned into pCEP4 at the unique *Xho* I restriction enzyme site, downstream from a human cytomegalovirus (CMV) immediate-early gene enhancer-promoter. pCEP4 is an episomal mammalian expression vector that uses the CMV immediate early enhancer/promoter for high level transcription and expression of recombinant genes inserted into the multiple cloning site. pCEP4 is an Epstein-Barr Virus (EBV)-based vector. Its replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) allow the plasmid to be maintained extrachromosomally and, offer high-copy episomal replication in human, primate and canine cells. pCEP4 carries the hygromycin B resistance gene for stable selection in transfected cells. The ColE1 origin of replication and ampicillin resistance gene allow for growth and selection in *E. Coli.*

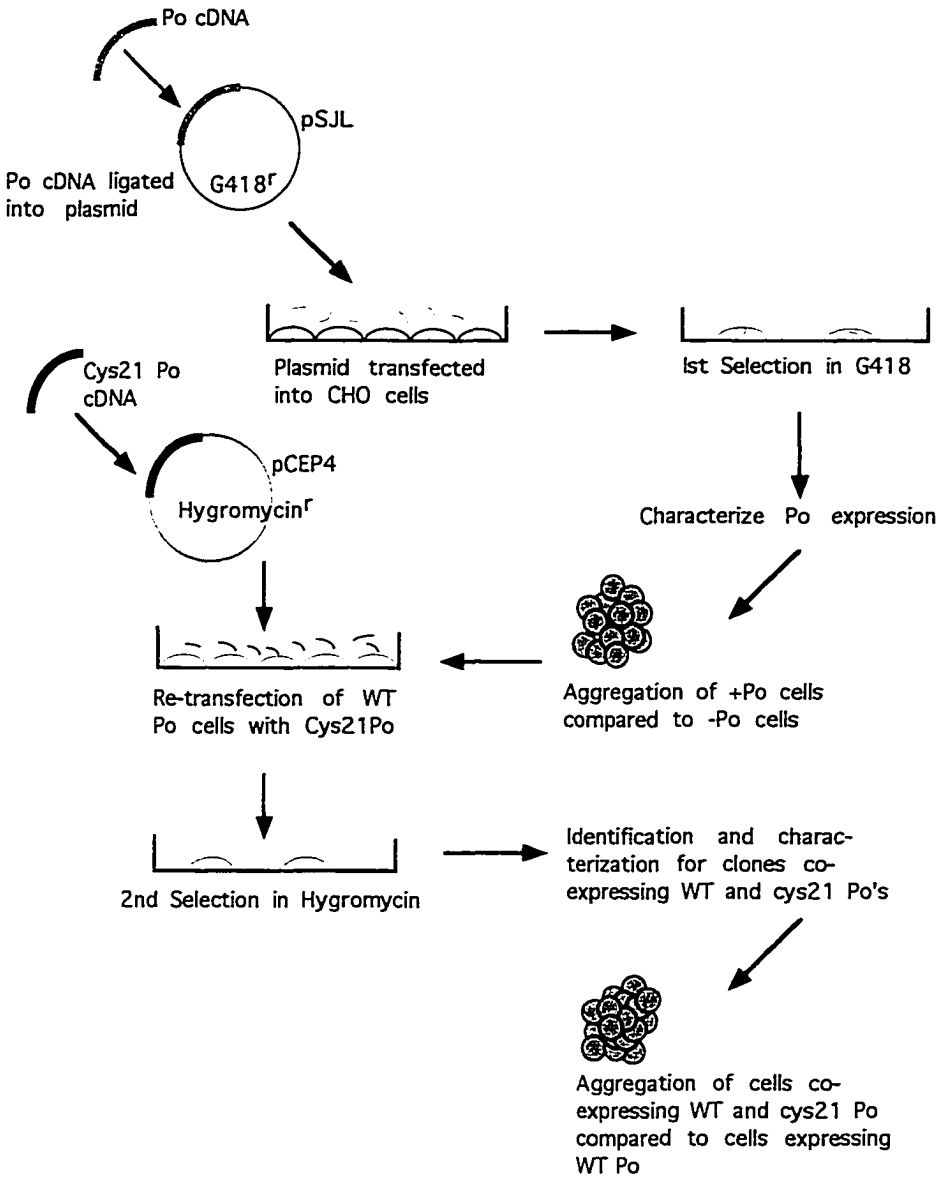


Figure 23: Schematic diagram of procedures to co-express wildtype and Cys21-mutated Po in CHO cells. Plasmid containing the wildtype Po-cDNA were transfected into CHO cells by calcium phosphate precipitation. Transfectants were selected initially in G418 and then for amplification by the methotrexate-dihydrofolate reductase strategy. After the expression of Po protein was documented at the cell surface, the adhesive properties of the cells expressing Po were compared to that of the control cells not expressing Po. Cells expressing the wildtype Po protein were re-transfected with the cDNA for Po mutated at Cys21 in the vector pCEP4 and, selected in 300 μg/ml hygromycin. A number of hygromycin resistant clones were picked and characterized for co-expression of the wildtype Po and Cys21-mutated Po proteins. The adhesive properties of co-expressor cells, cells expressing only the wildtype Po and control cells not expressing Po protein were compared using adhesion assay.

Characterization of Cells Expressing Both Wildtype and Mutated Po

The cells expressing both the wildtype and mutated Po, the co-expressers, were examined for their expression of both the wildtype and mutated Po proteins on the surface using immunofluorescence staining of fixed, unpermeabilized cells (see Chapter II Materials and Methods). In addition, surface expression level was quantitated using an ELISA (see Chapter II Materials and Methods). The adhesive properties of the co-expressers were assessed and compared to CHO cells expressing only the wildtype Po protein and control CHO cells by the adhesion assay described in Chapter II Materials and Methods.

Results

Co-expression of Wildtype Po and Cys21-Mutated Po in CHO Cells

To determine if Po mutated at Cys21, a non-adhesive protein, was able to interfere with the adhesion of wildtype Po and have a dominant-negative effect, a cell line already expressing wildtype Po was re-transfected with the cDNA of C21A Po carried on pCEP4, a vector resist to hygromycin (Fig. 22 & 23). Hygromycin-resistant colonies were picked and screened for co-expression of wildtype and mutated Po. This was done by assessing Po expression of the co-transfected cells by Western blotting, under reducing and non-reducing conditions.

Under non-reducing conditions the wildtype protein should be present in an oxidized form with a lower apparent molecular weight of about 28 kD because of its intact disulfide bond (Fig. 24). Under reducing conditions, the disulfide bond of wildtype Po is opened up. The apparent molecular weight of the wildtype is somewhat larger at about 30 kD. On the other hand, the Cys21-mutated Po protein, will be present a higher apparent molecular weight of 30 kD under both reducing and non-reducing conditions. This is because under the same conditions, Cys21-mutated Po protein will exist in a linear form, due to the lack of the disulfide bond. Therefore, under non-reducing conditions, cells expressing

only wildtype Po will have only one band of 28 kD, while a co-expressor will have two bands, the 28 kD (the wildtype Po protein) and the 30 kD (the Cys21-mutated Po protein). Using this criterion of co-expression, three clones from the re-transfection were chosen for further study, clone #8, 18 and 24. Clone 8 and clone 24 corresponding to lane e, f and lane g, h in Fig. 25 were co-expressors. On the other hand, clone 18 expressed only wildtype Po protein, because it had only one band of an apparent molecular weight of 28 kD under non-reducing conditions.

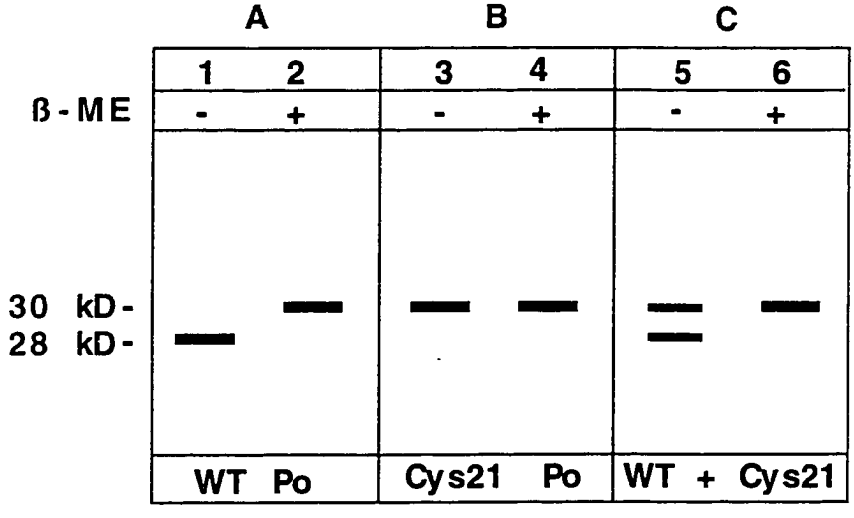


Figure 24: Schematic diagram of expected results of Western blot for cells co-expressing the wildtype and Cys21-mutated Po proteins in the presence and absence of the reducing agent, ß-mercaptoethanol (ß-ME). **Lane 1, 3** and **5** are without ß-ME. **Lane 2, 4** and **6** are treated with ß-ME. **Panel A** represents cells expressing only the wildtype Po protein. There will be a shift of Po's molecular weight from 28 kD to 30 kD in the absence and presence of ß-ME. **Panel B** predicts cells expressing only Cys21-mutated Po protein. The molecular weight of the mutated Po will remain about 30 kD under both reducing and non-reducing conditions. **Panel C** resembles cells expressing both the wildtype and Cys21-mutated Po proteins. There will be two bands under non-reducing condition, the 28 kD wildtype Po and the 30 kD Cys21-mutated Po protein.

To quantitate the surface expression of Po in the co-expressors, an ELISA assay on fixed, unpermeabilized cells of the three clones was carried out (Fig. 26). The expression of the co-expressors, clone 8 and clone 24, were shown to be comparable to that of clone 18, the clone only expressing the wildtype Po. This indicates that there is equivalent amount of Po expressed on the cell surface in the three cell lines.

Adhesion of CHO Cells Co-expressing Wildtype Po and C21A Po

The three clones of cells were subjected to the re-aggregation adhesion assay as were the cells expressing only wildtype Po protein. As we had previously reported, by 60 minutes, the cells expressing the wildtype Po had formed large aggregates and the total particle number had dropped, an indication of aggregate formation (Fig. 27). Similarly, for

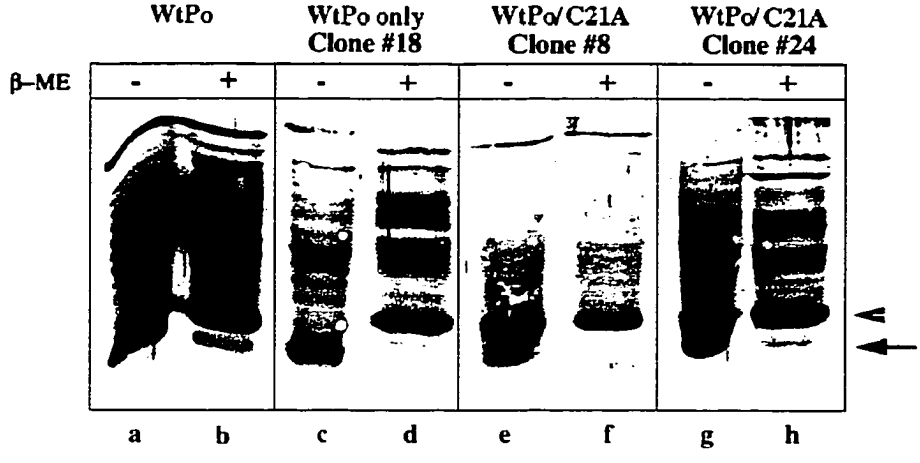


Figure 25: Western blot immunostained for Po of cells expressing wildtype Po only (**lanes a-d**) or wildtype Po and Po mutated at Cys21 (**lanes e-h**). Cells in **lanes a** & **b** are resistant to only G418 (one transfection) and cells in **lanes c - h** are resistant to both G418 and hygromycin (two transfections). **Lanes a**, **c**, **e**, and **g** are without ß-mercaptoethanol, **lanes b**, **d**, **f** and **h** are with ß-mercapoethanol. Arrowhead points to reduced Po. Arrow points to unreduced Po. 30 μg total protein per lane.

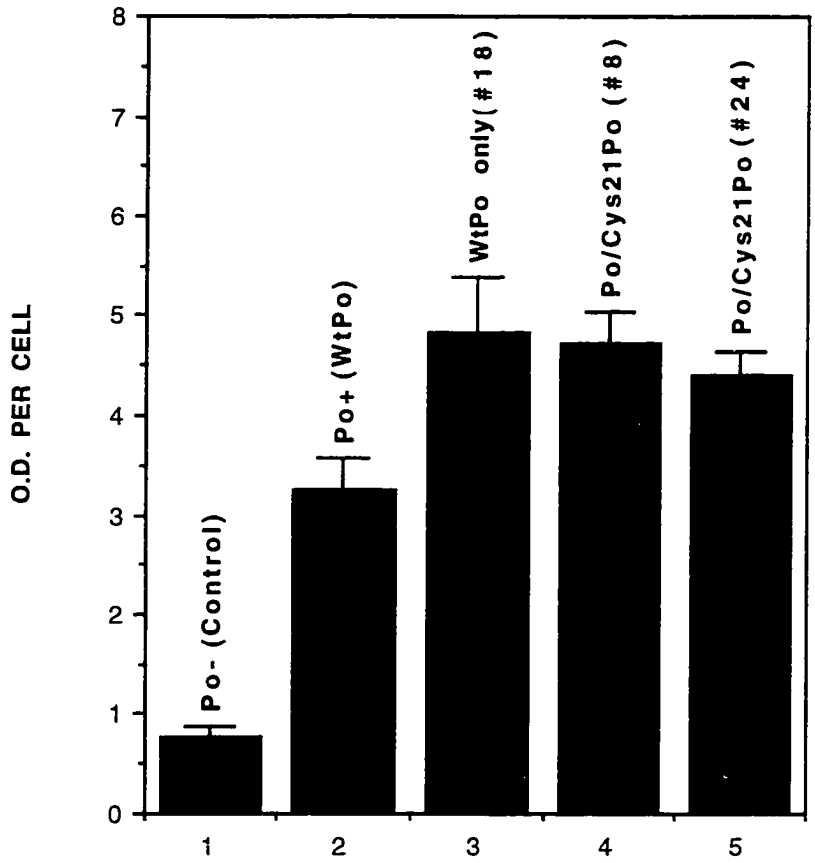


Figure 26: Quantitation of Po expressed at the surface of CHO cells co-transfected with wildtype Po and Cys21-mutated Po. The relative amount of Po expressed at the cell surface was quantitated by an ELISA of **1**, control transfected cells (Po-); **2**, cells expressing wildtype Po (Po+); **3**, hygromycin resistant cells expressing only wildtype Po, clone #18; **4&5**, hygromycin resistant wildtype Po and Po mutated at Cys21 (Po/Cys21Po) co-expressor cell, clone #8 and 24, using a Po-peptide antibody directed against sequences in the extracellular domain. Results are expressed (+/- SE) in relative absorbance units per cell and are the mean of three experiments, 40 samples per experiment.

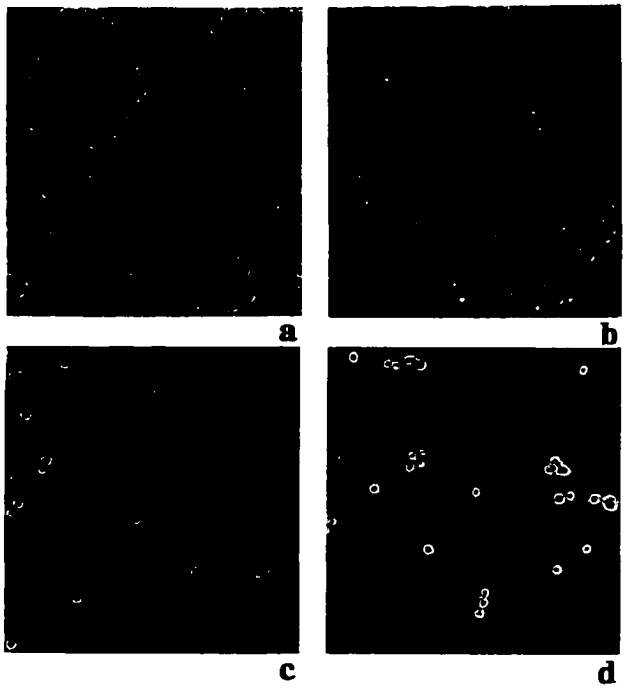


Figure 27: Aggregation properties of cells co-expressing wildtype Po and Cys21-mutated Po examined under microscope. Single-cell suspensions of CHO cells expressing (**a**) wildtype Po, (**b**) hygromycin resistant with wildtype Po, clone #18, (**c**) and (**d**) wildtype Po and Po-mutated at Cys21 co-expressor, clone #8 and #24. Samples were withdrawn at intervals and examined under the microscope. Results shown are after 120 min incubation.

clone 18, the cells expressing only wildtype Po protein, also formed large aggregates and the total particle number dropped to 20% of the starting value by 60 min (data not shown). In contrast, the co-expressors, both clone 8 and clone 24 each expressing wildtype and Cys21-mutated Po, failed to form large aggregates in the same time, and the total particle number had dropped to only about 80%, a value undistinguished from control cells not expressing Po. When the percentage of drop in the total particle number of the cells expressing only the wildtype Po was calculated to estimate the specific binding, the specific binding for clone #18, the clone expressing only wildtype Po, was found to be 100%; for clone #8 and 24, the co-exprssors, adhesion did not occur (Fig. 28). This suggests that the presence of Cys21-mutated Po protein interferes with the adhesive function of the pre-existing wildtype Po protein.

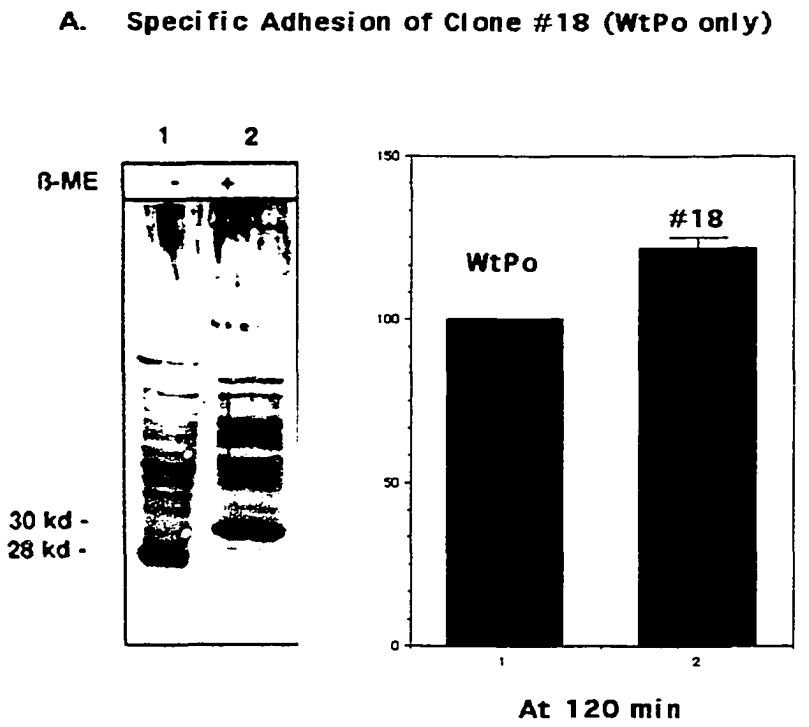
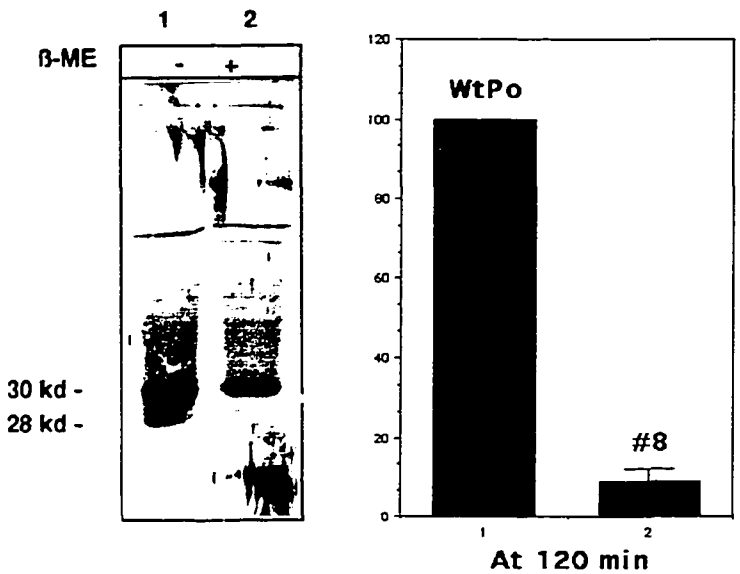
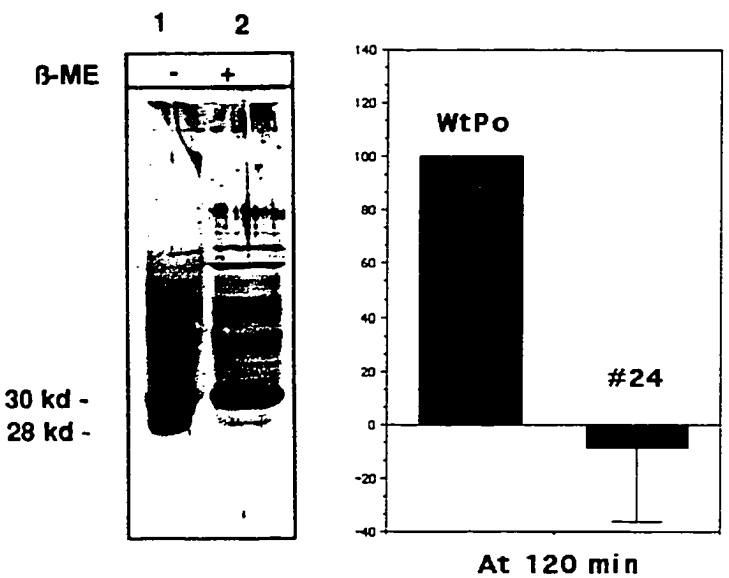


Figure 28: Loss of Po-mediated adhesion of CHO cells by co-expression of wildtype and Cys21-mutated Po. Aggregation was allowed to proceed as described in Fig. 27. At various intervals, samples were withdrawn and the total particle number was counted in a Coulter counter. Specific binding was calculated as the percentage drop in total particle number of the cells expressing only the wildtype Po. (**A**), cells hygromycin resistant but only expressing wildtype Po, clone #18. (**B**) & (**C**), specific adhesion of clone #8 and 24, co-expressors of wildtype and Cys21-mutated Po. Results presented are specific adhesion (+/- SE) after 120 min incubation and represent the mean of at least 3 experiments, each carried out in duplicate.

B. Specific Adhesion of Clone #8 (WtPo/Cys21)



C. Specific Adhesion of Clone #24 (WtPo/Cys21)



Summary

1. Clones 8 and 24 co-expressed the wildtype and Cys21-mutated Po proteins, while clone 18 did not express the mutated Po.

2. The level of surface expression of Po by clones 8 and 24 was equivalent to that of clone 18 expressing only the wildtype Po.

3. In the adhesion assay, clone 18 formed large aggregates by 60 minutes and its total particle number dropped to 25% of its starting number. In the same time, the two co-expressors, clones 8 and 24, had not formed large aggregates and the total particle number dropped to only about 80% of the original number.

4. When presented as a percentage of specific adhesion, with 100% adhesion taken as adhesion of the cells expressing only the wildtype Po, the co-expression of the Cys21-mutated Po abolished the adhesion of the wildtype Po completely.

Chapter V

Discussion and Conclusion

Part I

The Structure of Po-ED

Based on the analysis of a rat Po cDNA clone (Lemke and Axel, 1985) and results from the direct amino acid sequencing of bovine Po (Sakamoto et al., 1987), Po consists of 220 amino acids, with 124 residues in its extracellular N-terminal region. It was also suggested that Po has a single transmembrane domain of 26 residues (amino acids 125-150) and a cytoplasmic domain of 69 residues. Within its extracellular region, Po contains an Ig-like domain (Lai et al., 1987; Lemke and Axel, 1985). Po is N-glycosylated at Asn93, within its Ig-like domain (Fig. 2 & 3) (Lemke and Axel, 1985; Sakamoto et al., 1987).

Although many observations have indicated that Po is a homophilic adhesion molecule involved in myelin compaction, the exact functional domains are not known. In addition, Po has been suggested to be the closest molecule to the ancestral gene of the Ig superfamily (Barclay et al., 1987; Williams and Barclay, 1988). Hence, mapping its functional regions in the Ig domain of Po may have a broader implication to the Ig superfamily. To map Po's functional domain, we planned a systematic analysis on Po's Ig-like domain. Previous studies from us and others have indicated that both carbohydrate and the protein backbone play a role in

Po's adhesion (Filbin and Tennekoon, 1991; Filbin and Tennekoon, 1993; Schneider-Schaules et al., 1990; Griffith, 1992).

The Role of Po-ED's Oligosaccharide in its Adhesion

Our previous study lead us to conclude that Po must be glycosylated for the adhesion to occur. However, whether the carbohydrate moieties are directly involved in the adhesion remains unknown. In this respect, taking together studies from us and others (Schneider-Schaules et al., 1990; Griffith et al., 1992; Yazaki et al., 1992), we suggested that, rather than being directly involved in the adhesion, the carbohydrate in Po plays a role in positioning Po's Ig-like domain in an optimal orientation relative to the membrane, so that Po can interact with the Po molecule in the opposing membrane. Our suggestion fits into the predicted three-dimensional structural model of Po's Ig-like domain (Wells et al., 1993).

In this predicted model of Po's Ig like domain, the carbohydrate of Po was predicted to be located at the bottom of the Ig-like domain, holding up the Ig fold for interaction with the opposing Po molecule. If the sugar is removed, the model predicted that the Ig-like domain would collapse back onto its cell membrane and fail to interact with the opposing molecule (Wells et al., 1993). This location of the glycosylation site Asn93 is

confirmed in Po's crystal structure. In Po crystals, Asn93 is located in the base of the molecule near the cell membrane (Shapiro et al., 1996). As we postulated that the carbohydrates of Po are involved in properly positioning Po's Ig-like domain, we decided to focus our search for Po's functional domain on the molecule's protein backbone. To map precisely the adhesive regions of the protein backbone of Po-ED, we tested the ability of both antibodies against peptides found in the Po-ED and the peptide themselves to inhibit adhesion. In addition we studied in more detail the Po peptide 91-95 (SDNGT) by site-directed mutagenesis.

The Role of Po-ED's Protein Backbone in its Adhesion

To map precisely the functional region in Po's Ig-like domain, antibodies against three Po peptides were raised and tested for their ability to inhibit adhesion. In addition, the three peptides themselves, the Po 38-46, the 74-82 and 91-95 were also used to test their ability to inhibit the adhesion of Po molecule. The choice of these amino acid sequences from the extracellular domain was based on their high hydrophilicity based on a hydrophobicity plot of Po's extracellular domain, as hydrophilic residues are more likely to be exposed on a folded molecule's surface and are therefore to be in a position to interact with another Po molecule. In addition, after the primary sequence of Po protein folds into its tertiary

configuration, the peptides chosen will probably fall in the turns between the antiparallel ß-strands of the Ig domain. As the amino acid sequences within the loops are more variable than those of the ß-strands in an Ig domain, the amino acids at the turns have been proposed to play a role in directly interacting with other molecules while the sequences comprising the ß-strands of an Ig domain are suggested to be involved in conformational stabilization (Williams and Barclay, 1988).

The adhesion inhibition assay with the antibodies against the three peptides, Po38-46, 74-82 and 91-95 (SDNGT), showed that peptide SDNGT is important for the homophilic adhesion of cells expressing Po protein (Fig. 8). As a control pre-immune serum and an antibody against a cytoplasmic sequence of Po were used. Our results showed that none of these controls inhibited Po's adhesion.

The specificity of the inhibition by antibody against Po peptide 91-95 was further tested by using the peptide itself in the adhesion assay since small peptides unlikely exert a steric hindrance effect. As with the antibody, the homophilic adhesion of CHO cells expressing Po protein was completely blocked by the peptide SDNGT (Fig. 9 & 10). Hence, the possibility that the antibody's inhibition on adhesion is a result of its steric hindrance is unlikely.

Taken together the results of adhesion inhibition by the antibody against peptide SDNGT and inhibition by the peptide itself, suggests that Po91-95 is directly involved in Po's adhesion. This suggestion is further supported by the loss of Po's adhesion when two of the residues in this peptide, Asp92 and Gly94, are mutated. Through site-directed mutagenesis and our transfection/adhesion system, we showed that when Asp92 and Gly94 of Po were changed to Glu92 and Ala94, respectively, the transfected CHO cells were not able to adhere homophilically. The loss of the adhesion of Po mutated at Asp92 and Gly94 is unlikely to be a result of a conformational disruption of the folding of Po as both mutations are conservative substitutions. Furthermore, the mutated Po was glycosylated to the same extent as wildtype Po and reached the cell surface, strongly suggesting conservation of the mutated Po's tertiary structure.

For studies with Po74-82, both inhibition by the antibody against Po74-82 and by the peptide itself, suggest that this Po sequence is partially involved in Po's adhesion. It is possible that Po74-82 represents an adhesive determinant unrelated to the SDNGT determinant. In addition, the determinants of Po molecule may consist of nonconsecutive peptide sequences, Po74-82 may represent only part of a functional determinant as its inhibition of adhesion never reached 100%.

It has been proposed that Po molecules on the cell surface need to cluster/oligomerize in the same cell membrane (a *cis* interaction) in order to interact with opposing Po molecules (a *trans* interaction) (Wong and Filbin, 1996). Whether Po SDNGT and Po 74-82 are involved in a *cis* or *trans* interaction of Po molecule is difficult to test. A model of the tertiary structure of Po has been proposed by Wells et al. (1993). This model was based on the crystal structure of the VH-domain of phosphocholine-binding immunoglobulin M603 of mouse. According to the model, amino acids NGT of the SDNGT sequence are exposed at the surface of the Po molecule, in a position suitable for either *cis* or *trans* interaction. On the other hand, in this model, Po 74-82 appears to be in a position more favorable for a *trans* interaction. Furthermore, when the crystal structure of Po was solved, these predictions gain support from studies on the crystal of Po's extracellular domain.

In the model based on the crystal structure of Po (Shapiro et al., 1996), Po 74-82 is in the loop between the D and E strands and near the apex of the Ig domain. The side-chain of residue Trp78 points outward, and could interact directly with the negatively-charged lipid headgroups in the opposing membrane. Po 91-95 (SDNGT) is at the beginning of the F strand, close to the base of the molecule, near to the putative cell membrane. Therefore, Po 91-95, together with its attached carbohydrate,

may play a role in holding the molecule at the base of the Po molecule near the cell membrane. In addition, Asn93 is about 10 Å away from the closest adhesive interface. It is suggested that the carbohydrate could be involved in a *cis*-interaction with a near-by Po molecule (Shapiro et al., 1996; Griffith et al., 1992).

Part II

The Stabilization of Po's Ig-like Domain

The 2 ß-sheets of an Ig-like domain are believed to be stabilized into a sandwich-like structure by the interaction between the hydrophobic side chains of the ß-strands. Such a structure is thought to be further strengthened by the formation of a disulfide bond between two cysteines conserved in most Ig-domains. As a result, the Ig-like domain is folded into a compact, globular structure that is proteolytically resistant (Hunkapiller and Hood, 1989). However, such a role for the disulfide bond in an Ig-like domain's stabilization had never been demonstrated for any member of the Ig superfamily.

Po is an ideal molecule to address the role of the disulfide bond in an Ig-like domain as it possesses only one disulfide bond in its extracellular domain. Such a study was made feasible through the transfection/adhesion assay system we have already established (Filbin et al., 1990). In this system, the disulfide bond of the Po cDNA can be disrupted by altering one of the two cysteines of its disulfide bond prior to the transfection into CHO cells. After the mutated Po is expressed, changes in the adhesion of the Po devoid of its disulfide bond can be

directly monitored by an adhesion assay of CHO cells expressing the mutated Po.

Po's Disulfide Bond is Essential for its Conformation and Adhesion

Our adhesion assay results suggest that the transfection of CHO cells with Cys21-mutated Po failed to increase the cells' adhesive ability (Fig. 21). The loss of Po's adhesion after its disulfide bond is disrupted, indicates that Po's disulfide bond is required for its adhesion. Such a result is not a surprise as the disulfide bond in an Ig-like domain has long been suggested to be involved in the stabilization of the structure of an Ig-like domain (Williams, 1982; Williams and Barclay, 1988; Hunkapillar and Hood, 1989). Therefore, when the disulfide bond was removed, the conformation of the two ß-sheets in relation to each other may be altered. Nevertheless, the structures within each of the ß-sheets may still be maintained, as the Cys21-mutated Po protein was still able to be glycosylated. This, in turn, suggests that the mutated protein retained some features of its original structure, such that its glycosylation site was not masked. Another indication of the partial preservation of the mutated Po's original structure is that, the mutated Po was not misfolded to such an extent that it was held in the endoplamic reticulum, as it could be detected at the cell surface in our experiments.

Despite the loss of the adhesive function of Po with a partial preservation of its original conformation, after the disruption of its disulfide bond could indicate that the adhesive domain of Po consists of non-consecutive amino acid sequences from its two ß-sheets. The absence of the disulfide bond could cause an alteration in the positioning of the two ß-sheets relative to each other. So continuous adhesive determinants could no longer be formed if its constituent residues are from the two separate structures.

In a few members of the Ig-like superfamily the conserved disulfide bond is absent (Williams and Barclay, 1988). In these molecules, the cysteine residues are often replaced by an hydrophobic amino acid. Perhaps, the stabilization of such an Ig-like domain occurs because of extra strength of interactions contributed from those hydrophobic residues that replace the cysteines. Nevertheless, the disulfide bond in Ig-like domains is highly conserved and present in the majority of the Ig-like superfamily.

Therefore, although the Ig-like superfamily consists of a large number of molecules with diverse, specific functions, the tertiary structure of an Ig-like domain is highly preserved among the members of this family of molecules. Studies on these conserved features of the Ig-like superfamily may be pertinent to the whole group of molecules. Our work

with Po's disulfide bond suggests that Po's disulfide bond is essential for Po's homophilic adhesive function.

Part III

The Involvement of Po in HMSN

Since Po mutations were first linked to the development of CMT1B (Hayasaka et al., 1993; Kulkens et al., 1993; Su et al., 1993), there have been 29 mutations identified in *MPZ* of HMSN patients, with 22 mutations at 16 amino acids of Po's extracellular domain (Shapiro et al., 1996; Warner et al., 1996). The most prominent features of hereditary motor and sensory neuropathies (HMSN) are gradually progressive distal muscle weakness and atrophy (Chance and Pleasure, 1993). Although clinical symptoms overlap between different forms of HMSN (hereditary motor and sensory neuropathies), such as CMT1B, DSS (Dejerine-Sottas syndrome) and CH (congenital hypomyelination), patients with *MPZ* mutations present a wide spectrum of severity, ranging from the mild CMT1B to the more severe DSS and CH (Patel and Lupski, 1994; Chance and Fischbeck, 1994; Warner et al., 1996). The mechanism whereby the mutations in the gene of Po cause the heterogeneous forms of the disease, remains unclear.

Possible Dominant-Negative Effect of Mutated Po

One possible mechanism whereby a mutation in Po could cause the demyelinating disease HMSN, is the reduction in Po's gene dosage. Most

MPZ mutations in HMSN are heterozygous. Hence in these patients, the normal allele and a mutant allele for the gene of Po protein co-exist (Hayasaka et al., 1993; Kulkens et al., 1993; Warner et al., 1996). As such, at most only 50% of the normal levels of wildtype Po protein in HMSN is produced. That is to say, 50% of the Po protein produced by the mutated allele in these patients is aberrant and may not reach the cell surface. As a result, if a threshold level of wildtype Po in the cell membrane is required for effective homophilic adhesion to occur and the remaining 50% of the normal Po is below that level, then wildtype Po at half of the normal dose would fail to maintain myelin.

If the scenario of 50% of reduction in Po's gene dosage holds true for those mutations that produce Po proteins so mutated that they are unstable and never reach the cell surface, the phenotypic presentation of such mutations will resemble those of the heterozygous Po knock-out mice. These $Po^{+/-}$ mice show normal myelination until the age of 4 month. Then they develop a mild form of demyelination (Martini et al., 1995). Thus, the mild cases of HMSN such as CMT1B could represent the phenotypes of those mutations in *MPZ* that cause severe conformational changes in Po proteins.

A direct *in vivo* testing of such a hypothesis will need expression of alleles carrying the same mutations described in the patients, in transgenic

mice. A first step to producing transgenic mice will be to introduce such mutations into the wildtype Po cDNAs and transfect these altered Po cDNAs into CHO cells. The adhesive properties of such CHO cells would then be characterized and compared to those expressing the wildtype Po.

However, a decrease to half of Po's normal dose, alone, can not explain the severe cases of CMT1B and Dejerine-Sottas syndrome (DSS), which is a more severe form of HMSN with nerve conduction velocities less than 10m/s and an earlier onset (Dyck et al., 1993). A second possible mechanism whereby a mutation in the gene for Po would result in the demyelinating disease is that, the presence of the aberrant Po protein from the mutated allele may interfere with the function of the normal Po protein. In other words, the aberrant Po protein may have a dominant-negative effect on the wildtype Po molecule.

As most Po mutations reported in HMSN are scattered in the extracellular domain of Po, it is unlikely that these residues all participate directly in Po's adhesion. Instead, at least some of these mutations may cause a loss in Po's function by disrupting the conformation of the Po molecule. These aberrant Po molecules, with a change in their conformation, may then interfere with the wildtype Po in a dominant-negative manner. If this is the case, then the Po mutated at Cys21 (C21A Po) will provide a good model to study if the conformationally changed Po

molecules interferes with the adhesion of wildtype Po, since Cys21-mutated Po has been previously shown to be a non-adhesive protein that reaches the cell surface. As the disruption of its disulfide bond is likely to result in a conformational change, the loss of the adhesive function of the Cys21-mutated Po is likely to be a result of a conformational change rather than a failure to participate in adhesion directly. Therefore, Cys21-mutated Po is a good model for studying the putative dominant-negative effect of conformationally changed Po proteins on wildtype Po.

Despite the scenario of a dominant-negative effect, severe cases of HMSN could also be accounted for by homozygous mutations in *MPZ*. In two pedigrees (Ikegami et al., 1996; Warner et al., 1996) of HMSN, the parents which are heterozygous for mutations in the gene of Po presented as mild cases of CMT1, while the children, homozygous for the same mutations, have the more severe DSS. The difference in the phenotypic presentation of these heterozygous and homozygous mutations of *MPZ* is similar to those of heterozygous and homozygous mice for the null mutation of Po gene (Giese et al., 1992; Martini et al., 1995). The homozygous Po knock-out ($Po^{-/-}$) mice showed early and severe myelination deficiency while the heterozygous mice for the Po null mutation ($Po^{+/-}$) have normal myelination until 4 months of age. However, detailed mechanisms in regards to whether the aberrant Po proteins ever

reaches the cell membrane in these patients with homozygous *MPZ* mutations require further study.

Wildtype Po Does not Adhere when Co-expressed with a Mutated Po

By re-transfecting the cDNA of the Cys21-mutated Po into the CHO cells already expressing the wildtype Po (Fig. 23), we showed that, the expression of C21A Po abolished the adhesive function of the pre-existing wildtype Po in the same cell membrane (Fig. 28). Such results support the suggestion of a dominant-negative effect of an aberrant Po molecule. Similar effects have also been shown in Po truncated in the cytoplasmic domain (Wong and Filbin, 1996), as well as in other mutated adhesion molecules, namely cadherins (Kintner, 1992; Fujimori and Takeichi, 1993; Levine et al., 1994) and integrins (Balzac et al., 1994; Lukashev et al., 1994; Smilenov et al., 1994).

If there must be a critical number of wildtype functional Po molecules in the membrane in order for the effective membrane-membrane adhesion to occur, co-existence of non-adhesive Cys21-mutated Po in the same cell membrane could dilute the number of functional Po molecules in each cluster, presumably a tetramer (Shapiro et al., 1996). As a result, the sum affinity per tetramer would be diminished to an extent that an effective adhesion with the opposing/apposing cluster could no longer be

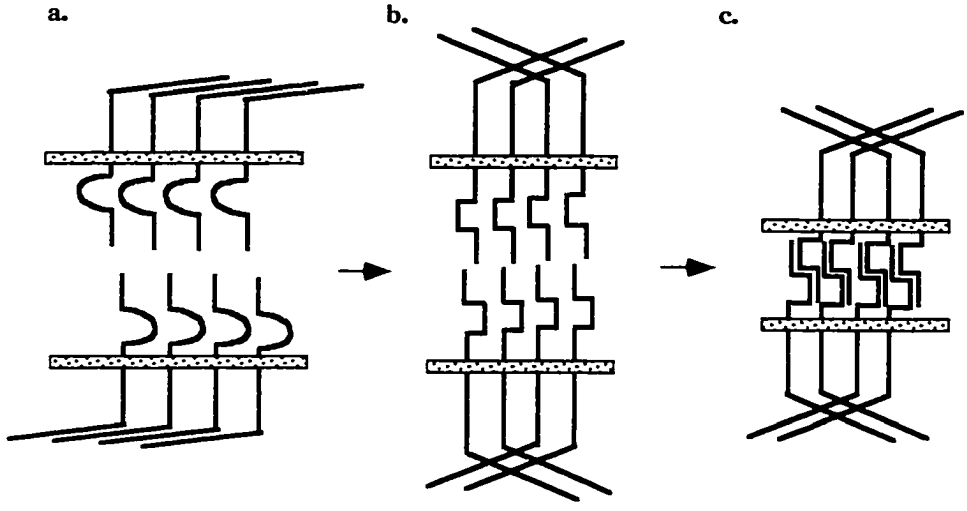
sustained, namely a dominant-negative effect of Cys21-mutated Po (Fig. 29). Such a scenario of a dominant-negative effect of Po mutated in the extracellular domain could explain some cases of CMT1B.

Previously it has been shown in our lab that the intact cytoplasmic domain is required for the adhesion of the extracellular domain of Po to take place, and that such adhesion is dependent on the interaction of the full-length Po, with the cytoskeleton (Wong and Filbin, 1994 and 1996). In addition, it was suggested that Po clusters with other Po molecules in the same cell membrane probably through an interaction between the cytoplasmic domain and the cytoskeleton.

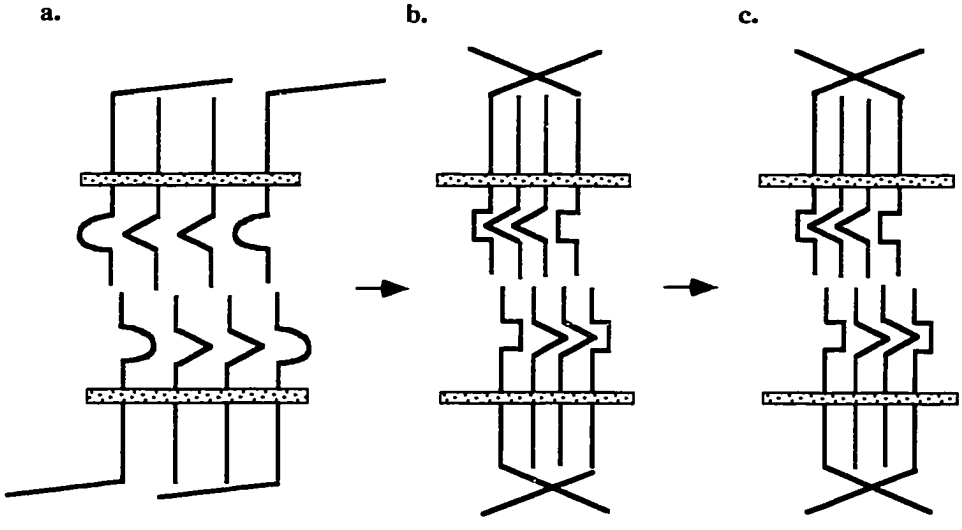
Another possible mechanism whereby Cys21-mutated Po exerts its dominant-negative effect on the co-expressed wildtype counterpart is that instead of creating a tetramer with a weaker affinity to adhere to the apposing membrane, the existence of the mutated molecule completely abolishes the formation of Po clusters. The absence of a *cis* interaction between the Po monomers in the cell membrane probably greatly diminishes Po affinity for the apposing Po molecule. It has been suggested that the affinity of individual adhesive molecules is generally weak (van der Merwe and Barclay, 1994; Shapiro et al., 1996) and that multimeric interactions between the monomers greatly increase the adhesiveness. The formation of Po tetramers requires direct interactions of nine residues

Figure 29: Model of a dominant-negative effect of Cys21-mutated Po on the adhesion of wildtype Po. **(A)** Interactions of wildtype Po when expressed alone. **(a)** An initial low affinity interaction of Po-extracellular domain with each other in a *cis*-manner and with cytoskeleton. **(b)** The initial interaction of Po-extracellular domain triggers a change in the interaction of the cytoplasmic domain with the cytoskeleton. The cytoskeleton re-organizes and pulls back, inducing a conformational change in the extracellular domain and clustering. **(c)** Both clustering and the conformational changes strengthen the adhesion of the extracellular domain. **(B)** Interactions of wildtype Po when co-expressed with Cys21-mutated Po. **(a)** An initial interaction of Po-extracellular domain with each other in a *cis*-manner and with cytoskeleton. **(b)** The initial interaction of Po-extracellular domain triggers a change in the interaction of the cytoplasmic domain with the cytoskeleton. The cytoskeleton re-organizes and pulls back, but fails to induce a conformational change in the extracellular domain of Cys21-mutated Po. **(c)** The overall adhesiveness is decreased because of the presence of the non-adhesive Po mutated at Cys21. Adhesion is not strong enough to hold two membranes together.

A. Wildtype Po only



B. Wildtype Po and Cys21-mutated Po



between the B-C loop of one Po monomer with the C"-D and E-F loops of another. The loss of the disulfide bond of Cys21-mutated Po could disrupt the conformation of the molecule, so that the tetramer could no longer form. As a result, individual Po molecules with weak affinity fail to proceed the homophilic adhesion with the apposing Po protein.

Although Cys21 of Po has never been found to be affected in HMSN, the mechanism of the dominant-negative effect of Cys21-mutated Po in our experiments could also be due to the existence of the free sulfhydryl group (-SH) of Cys98. The disulfide bond is formed between Cys21 and Cys98 in Po. The substitution of Cys21 with alanine frees the sulfhydryl group of Cys98 from its previous bonding. Hence the -SH group is free for bonding. The Cys98 from one Po monomer would presumably be able to form a disulfide bond with the Cys98 of another Po. The formation of such a dimer could affect the effective formation of the tetramer, hence homophilic adhesion is prevented from happening. Nevertheless, our studies show that when separated on a gel under non-reducing conditions, Cys21-mutated Po protein does not exist as a dimer. Therefore, it is unlikely that Cys21-mutated Po produces a dominant-negative effect by its free -SH group of Cys98.

Part IV

The Interactions between Po Protomers in Myelin Membrane

In an effort to map the functional regions of Po's extracellular domain, our adhesion inhibition results with antibodies against three Po peptides and the peptides themselves, namely the Po 38-46, the 74-82 and the 91-95, suggests that Po 91-95 may directly participate in Po's adhesion, Po 74-82 may partially participate whereas Po 38-46 probably is not involved in the adhesion. Based on its crystal structure, the primary sequence of an individual Po molecule, or a Po protomer, is modeled to form ten ß-strands, which in turn fold into two ß-sheets (Shapiro et al., 1996) (Fig. 30), as predicted before (Lai et al., 1987, Lemke et al., 1988), a structure typical of the Ig-superfamily .

It is suggested that every four Po protomers in the same cell membrane interact and form a ring-like tetramer (Shapiro et al., 1996) (Fig. 31). The tetramers in one cell membrane interact with the cyclic tetramers emanating from the opposing membrane, resulting in Po's homophilic adhesion (Fig. 32). The crystal structural data show that the B-C loop (Po 25-32) of one Po protomer, interacts *in cis* with the C"-D (61-69) and E-F (86-92) loops of the next Po molecule, resulting in the formation of a

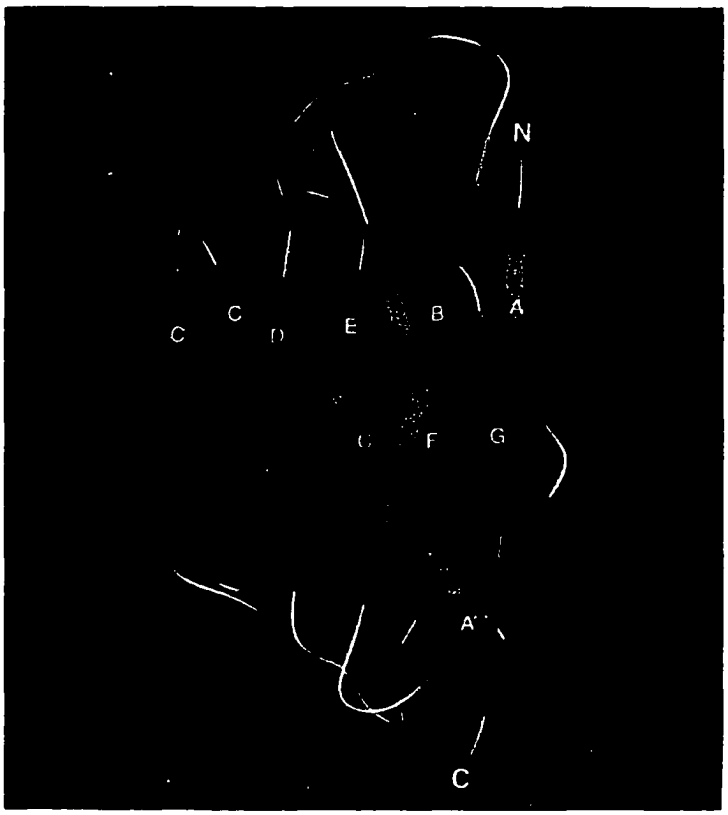


Figure 30: Ribbon diagram of Po's extracellular domain. Each ß strand is labeled. The residue boundaries of the ß strands A, A', B, C, C', C", D, E, F, and G, respectively, are 2-4, 8-11, 17-24, 33-40, 47-54, 57-60, 70-73, 82-85, 93-101, and 109-118. A hypothetical path for disordered residues 103-106 in the F-G loop is shown in grey. Modified from Shapiro et al., 1996 with permission.

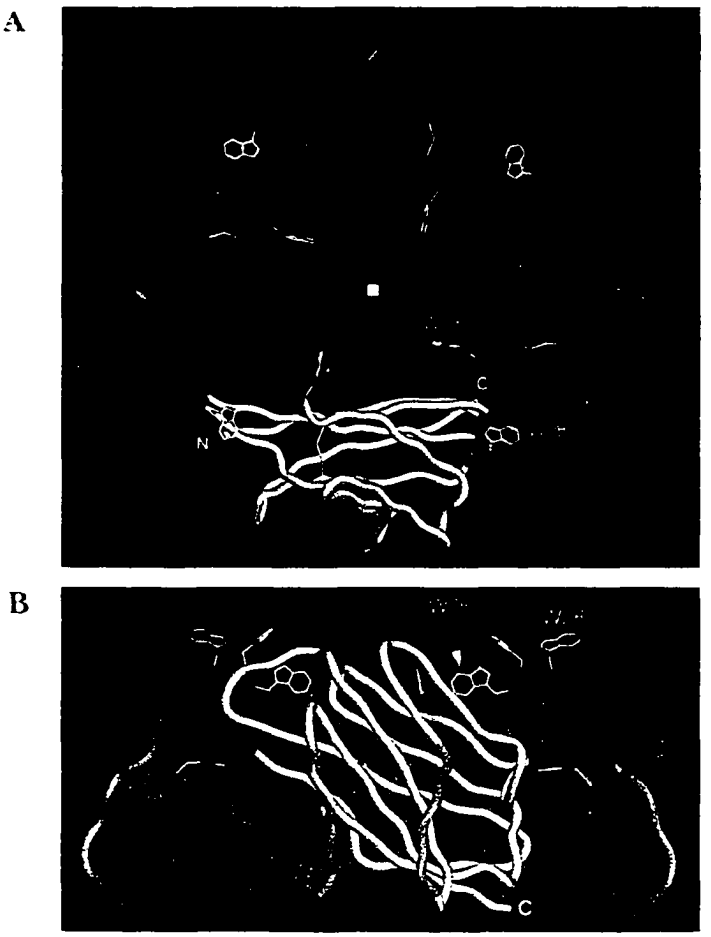


Figure 31: Molecular associations observed in crystals of Po's extracellular domain. (**A**) Fourfold doughnut. Cα worms are shown for each symmetry-related molecule. The position of the fourfold axis is indicated by a square. The outwardly pointing tryptophan side chains are shown. (**B**) Perpendicular view, with the fourfold axis vertical. All four protomers in each cyclic tetramer are in the same head-to-tail orientation about the fourfold axis. The C-termini are indicated pointing down out of the doughnut, while the N-termini are found midway through it. The side chains of Trp28 and Trp78 are labeled for the protomer on the right; the tryptophan side chain that seems to come between these two is from the protomer in the back. Modified from Shapiro et al., 1996 with permission.

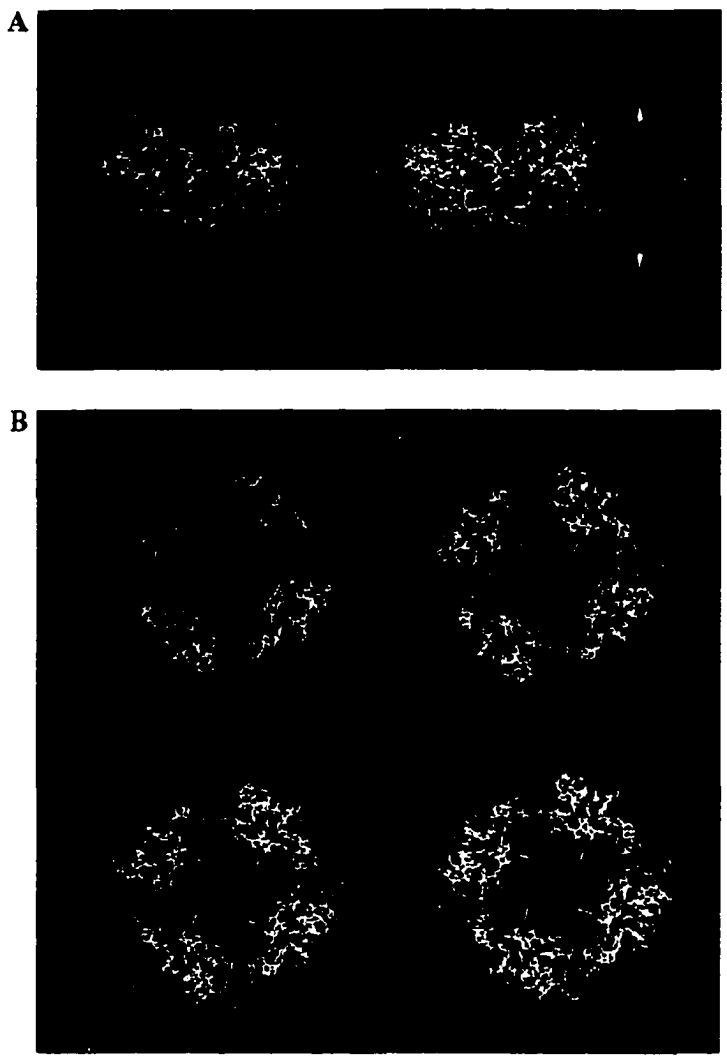


Figure 32: Combined Po interaction observed in the crystal lattice of Po's extracellular domain. The Po crystals are composed of layers of protomers. Each layer consists of alternately oriented cyclic tetramers. (**A**) The tetramers of Po's extracellular domain from two apposed membranes are joined by the putative adhesive interfaces for a *trans* adhesion between the two membranes. These tetramers are depicted here as they might emanate from their respective membrane surfaces (schematically indicated with dotted lines) at the C-termini, with the grey-colored tetramers protruding from the upper surface, and the white-colored tetramers from the lower surface. A model for main chain atoms for the five residues of the disordered linker to the membrane are shown in grey. (**B**) Perpendicular view of the layer of the crystal lattice. Each tetramer is related to four others of opposite head-to-tail orientation. Reprinted from Shapiro et al., 1996 with permission.

tetramer. Nine residues are proposed to be directly involved, namely, Val26, Tyr4, Gln16, Ser26, Glu68, Gln71, His86, and Asn87.

On the other hand, a different region of the Po protomer, the C' strand (Po 47-54) is proposed to be involved when a tetramer adheres with a partner tetramer from the opposing membrane. The residues that are involved in the adhesive interaction between the opposing tetramers include: Arg38, Arg45, Asp46, Ala47, Ser49, His52, Val107 and Gln112. In addition to the adhesion between the C' strands of the opposing Po tetramers, it is also suggested that the side-chains of Trp28 and perhaps Trp78 at the apices of the B-C loop (Po 25-32) and D-E loop (74-81) respectively, interact directly with the opposing membrane lipid bilayer, helping specify the spacing between the membranes.

The Adhesion Inhibition Results of Po Sequence 91-95 vis-à-vis Po's Crystal Structure Studies

According to the crystal structure, the glycosylation site of Po, Asn93, is at the beginning of the F strand (Po 93-101) (Fig. 30), located at the base of the molecule, near the cell membrane. Asn93 is about 10 Å from the tetramer-tetramer adhesive interface. However the sugar residues are thought to directly participate in the adhesion between the opposing tetramers. Po 91-95 (SDNGT) starts at the E-F loop (Po 86-92) and

extends into the beginning of the F strand (Po 93-101) (Fig. 30). The adhesion inhibition assays showed that both the antibody and the peptide of Po 91-95 inhibit Po's adhesion completely, indicating that the sequence is directly involved in the adhesion. Among the five amino acids of Po 91-95, except for residue Asn93, it is not mentioned in the report of the crystal structure whether residues Ser91, Asp92, Gly94 and Thr95 are directly involved in Po's adhesion (Shapiro et al., 1996). However, this does not exclude the possibility of a direct involvement of Po 91-95 in either the formation of a tetramer in the same membrane, or the tetramer-tetramer adhesion between membranes. Conservative substitutions at residues Asp92 and Gly94 caused a loss of Po's adhesion. Since it is unlikely that such conservative mutations will disrupt the conformation of the Po molecule, the loss of the adhesion is probably due to the alteration of a functional determinant contributed either by Asp92 or Gly94 of Po 91-95. This in turn supports the notion of a direct involvement of Po 91-95 in the homophilic adhesion of Po molecule.

If indeed Po 91-95 is involved in Po's tetramer formation, a Po molecule with mutations in sequence 91-95 would be expected to exert a dominant-negative effect on the wildtype Po protein, as tetramers containing only wildtype Po will dramatically decrease. They will account for only one sixteenth of the normal level of the wildtype Po tetramers,

provided that the mutated protomer can still sequester the wildtype Po into the tetramer. The clinical manifestation of such mutations would be expected to be a severe form of CMT1B. Nevertheless, if mutated Po loses its ability to associate with the wildtype Po completely, the wildtype Po molecules may be able to form tetramers among themselves in the same membrane. In this case, there may be half the normal level of the wildtype Po tetramers present, a mild form of CMT1B would be predicted.

If however, peptide 91-95 directly participates in the tetramer-tetramer adhesion between the membranes, Po with mutation in sequence 91-95 would not be expected to have a dominant-negative effect on the wildtype Po, as only one sixteenth of the tetramers formed are made entirely of the mutated Po protomers, whereas the majority of the tetramers would contain wildtype protomer to various degrees. Provided that tetramers containing the mutated Po are still able to interact with the opposing tetramers, the clinical manifestation of a mutation in sequence 91-95 would be expected to result in a mild form of CMT1B. However, if the ability of Po to interact in a *trans* manner would be affected by the presence of any mutated Po protomers in a tetramer, a more severe form of clinical outcome may be expected. Nevertheless, to date, there have not been any Po 91-95 mutations reported in patients with CMT1B.

The kinetics of the formation and dissociation of the Po tetramer is not known. If Po protomers are in a constant state of association and dissociation, accompanied by a changing steric orientation relative to the membrane, then the antibody against Po 91-95 may have the access and bind to residues in sequence 91-95. Since sequence 91-95 is close to both the base of the molecule and the sugar residues, the binding of its antibody could interfere with the interaction between the sugar residues and the Po protein backbone. The proper positioning and orientation of Po relative to the cell membrane and to other protomers would be affected as well, hence resulting in a failure of Po to adhere homophilically. However, this scenario does not seem to be the case as not only the antibody but also the peptide itself can inhibit Po's adhesion.

The Adhesion Inhibition Results of Po Sequence 74-82 in Comparison to Po Crystal Data

Po 74-82 is in the entire D-E loop (Po 74-81) and the beginning of the E strand (Po 82-85) in the model based on Po's crystal structure (Fig. 30). In the crystal model, residues Arg38, Arg45, Asp46, Ala47, Ser49, His52, Val107 and Gln112 are proposed to be directly involved in the adhesion between the opposing tetramers. In addition, residues Trp28 and Trp78 on the apices of the B-C and D-E loops are also suggested to

participate in the *trans* adhesion of Po by interacting with the opposing membrane. If this is indeed the case, either the antibody or the peptide of Po 74-82 will mask residue Trp78 and prevent it from accessing the opposing membrane, hence weakening the adhesion of Po. This model is in agreement with our adhesion inhibition results, namely, both the antibody and the peptide of Po 74-82 are capable of partially inhibiting Po's homophilic interaction. Since a Po mutated at Trp78 would be expected to still form a tetramer with other wildtype and mutated protomers, such a mutation would have a dominant-negative effect on the wildtype Po and, clinically produce a severe form of CMT1B. Nevertheless, to date, there have not been any cases reported with a mutation at Trp78.

The Adhesion Inhibition Results of Po Sequence 38-46 vis-à-vis Po's Crystal Structure Model

Po 38-46 is in the end of the C strands (Po 33-40) and in the entire C-C' loop (Po 41-46) according to Po's crystal model (Fig. 30). Although it is the C' strand (Po 47-54) that is suggested to participate in the adhesion between the opposing tetramers, residues Arg38, Arg45 and Asp46 are suggested to be directly involved in the *trans* interaction between opposing Po tetramer partners. However, our adhesion inhibition assay

showed that neither the antibody nor the peptide of Po 38-46 could inhibit Po's adhesion. It is possible that the antibody we produced does not recognize residues at either end of the Po peptide 38-46, namely residues Arg38, Arg45 and Asp46. However, this hypothesis will not explain the inability of Po peptide 38-46 to inhibit adhesion. Perhaps, without Arg38, Arg45 and Asp46, the rest of the residues suggested to be directly involved in the *trans* adhesion, e.g. Ala47, Ser49, His52, Val107 and Gln112, as well as the membrane interacting Trp28 and Trp78, are still able to sustain an efficient homophilic adhesion. So far there has not been any report of mutation in the sequence of Po 38-46 in CMT1B cases.

In conclusion, our results and those of others (Shapiro et al., 1996; Warner et al., 1996), indicate that a large number of amino acid residues and sequences in Po's extracellular domain seem to be essential for Po's adhesive function. Since Po is a small, compact molecule and probably involved in some form of *cis* interaction in addition to its *trans* adhesion, it should not be surprising to find an increasing number of mutations scattered in Po's extracellular domain are being identified in various pedigrees of neuropathies, and at the same time demonstrated or predicted in the laboratory. Some of these residues may be critical to Po's structure, some to Po's *cis* association whereas others are involved in Po's *trans* adhesion. However, with more experiments in the laboratory and

observations in the clinic, the jigsaw puzzle of Po's magic adhesive ability to keep the myelin sheath wrapped around an axon will eventually be solved.

Chapter VI

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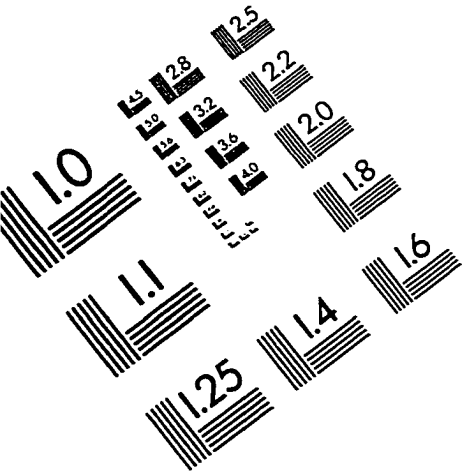
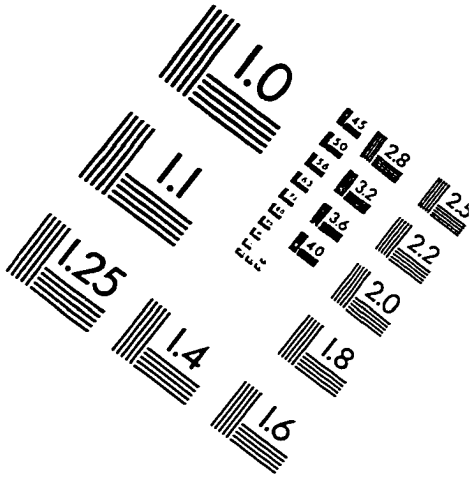
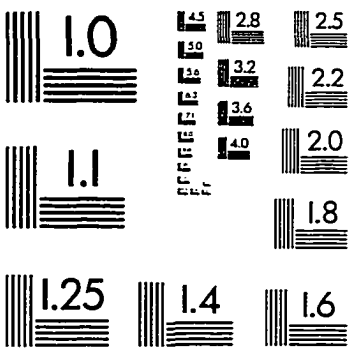
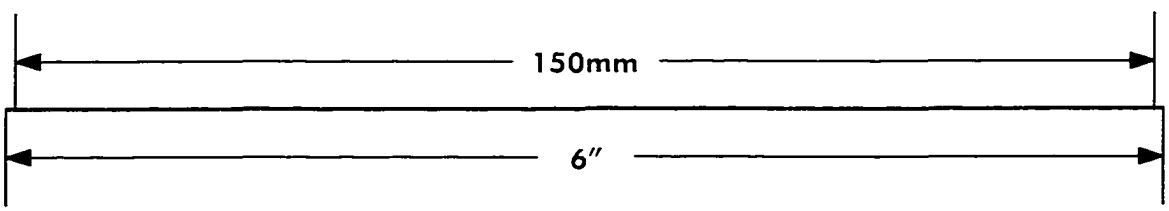
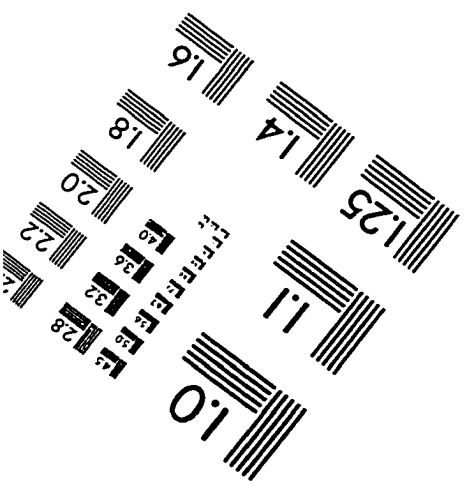
IMAGE EVALUATION TEST TARGET (QA-3)

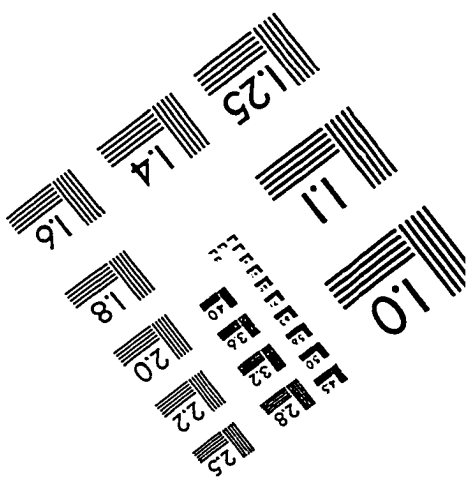












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