THE ROLE OF POST-TRANSLATIONALLY MODIFIED MYELIN PROTEINS IN THE MECHANISM OF DEMYELINATION AND THE ATTENUATION OF DISEASE BY PACLITAXEL IN THE ND4 TRANSGENIC MOUSE

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

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Abstract

Recent findings on myelin proteins isolated from Multiple Sclerosis (MS) patients and an animal model for demyelinating disease (ND4 transgenic mice) suggest that the mechanism of disease begins prior to the activation of the immune system and involves an alteration in the post-translational modification of the major myelin proteins. This defect may be inherent in oligodendrocytes, the myelin producing cells in the central nervous system, and give rise to a developmentally immature myelin that is more susceptible to attack. Using the ND4 mouse, the biochemical synthesis of the myelin proteins proteolipid protein (PLP) and its splice variant (DM20 protein) was examined. No differences were observed between normal and ND4 mice at the DNA or RNA level but a change was detected in the post-translational modification. Isolation and characterization of PLP and DM20 proteins from ND4 mice demonstrated an increase in the amount of covalently bound palmitate and stearate. Using chemically hyperacylated PLP, increased levels of covalently bound palmitate were shown to reduce the α-helical content of PLP and promote its interactions with lipid within a bilayer. Thus an elevation in covalently bound fatty acids in PLP and DM20 could alter the stability of the myelin bilayer. Subsequently, myelin may become more susceptible to proteolytic digestion which in turn can digest myelin proteins to produce encephalitogenic peptides. In treating demyelinating disease, several steps within the mechanism of demyelination must be targeted. Paclitaxel, a drug which could potentially act at several steps, was injected into ND4 mice and was found to reduce the severity and progression of clinical signs, inhibit proliferating astrocytes, and promote remyelination in the ND4 mice.

The findings reported in this thesis suggest one mechanism of demyelination which involves a defect in post-translational modification of myelin proteins, followed by formation of a less stable myelin and the response by other cells or components to this myelin. By targeting several different steps within this mechanism, paclitaxel was shown to be an effective drug in the treatment of demyelinating disease.
Acknowledgments

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For their love and support, I would also like to thank my sisters Teresa and Renée and my brother-in-law Roger.

To Laura, thank-you for your constant and valued encouragement, advice, companionship, and love.

Lastly, I want to thank my parents for giving me the opportunity to study for which I am deeply appreciative. I dedicate this thesis to my loving parent.
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<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAEE</td>
<td>benzoylarginine ethyl ester</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BF₁-1</td>
<td>oligo-1 binding factor 1</td>
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<td>BF₁-2</td>
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<td>Blotto</td>
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<td>cluster of differentiation adhesion molecule 44</td>
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<td>CM-Tris</td>
<td>carboxymethyl-tris acryl resin</td>
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<td>4-CN</td>
<td>4-chloro-1-naphthol</td>
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<td>CNS</td>
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<td>CREAЕ</td>
<td>chronic relapsing EAE</td>
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<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
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<td>ddH₂O</td>
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<td>ΔDM20</td>
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<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GC/MS</td>
<td>tandem gas chromatograph/ mass spectrometer</td>
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GFAP  glial fibrillary acidic protein
GMSA  gel mobility shift assay
GuSCN guanidinium isothiocyanate
Hepes N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H2O2 hydrogen peroxide
hPLP hyperacetylated PLP
hrs hours
H2SO4 sulfuric acid
Ig immunoglobulin
IPL intraperiod line
Jp Jimpy mouse
Jpmsd Jimpy myelin-synthesis deficient mouse
JpRsh Jimpy rumpshaker mouse
kb kilobase
KCl potassium chloride
kDa kilodalton
KH2PO4 potassium phosphate monobasic
M molar
MBP myelin basic protein
MBP-Cit 0 or MBP-C1 MBP component 1
MBP-Cit 6 or MBP-C8 MBP component 8 with 6 citrullinyl residues
MBP-Cit 18 Marburg-type MBP component with 18 citrullinyl residues
mdeg millidegrees
MDL major dense line
mg milligram
Mg2+ magnesium
MgCl2 magnesium chloride
MHC class II major histocompatibility complex class II
min minute
ml millilitre
mM millimolar
MMPs matrix metalloproteinases
Mn2+ manganese
MRI magnetic resonance imaging
mRNA messenger RNA
MRW mean residue weight
MS Multiple Sclerosis
mS milliSiemens
MyTi-1 myelin transcription factor-1
N2 proteolipid protein or lipophilin
Na-Acetate sodium acetate
Na-citrate sodium citrate
NaCl sodium chloride
Na2HPO4 sodium phosphate dibasic
NaOH sodium hydroxide
ND4 transgenic mouse line with 70 copies of DM20
ng nanogram
nm nanometre
nPLP naturally acylated PLP
OD optical density
PAD peptidylarginine deiminase
PBS  phosphate-buffered saline
PC  phosphatidyl choline
PITC  phenylisothiocyanate
PLP  proteolipid protein
pMD14  rat PLP cDNA
PMSF  phenylmethylsulfonyl fluoride
PNS  peripheral nervous system
Poly dI-dC·dI-dC  Polydeoxyinosinic-deoxycytidylic acid
pt  paralytic tremor rabbit
[θ]  mean residue ellipticity
[θ]_{obs}  observed residue ellipticity
RNA  ribonucleic acid
RNase A  ribonuclease A
rpm  revolutions per minute
S  order parameter
SDS  sodium dodecyl sulfate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SV40  simian virus 40 region
TBS  tris buffered saline
Tc  phase transition temperature
TIMP  tissue specific inhibitors of matrix metalloproteinases
Tris-HCl  tris hydroxymethyl aminomethane hydrochloride
μCi  microCurie
μl  microlitre
μM  microMolar
μg  microgram
V  volts
Preface

At the beginning of this project, the main goal was to determine the biochemical changes leading to and accompanying demyelination in a spontaneously demyelinating transgenic mouse model (ND4). As the mechanism of demyelination in this model became clearer, the focus of the project expanded to include treatment of disease. In order to communicate the findings from these studies, this thesis will be presented in two parts.

The first part outlines a mechanism of demyelination based on changes in the biosynthesis and the structure of the major myelin proteins proteolipid protein (PLP), DM20, and myelin basic protein (MBP). By correlating earlier findings with changes in post-translational modification of DM20 and PLP in ND4 mice presented in this thesis, a mechanism of demyelination was derived and summarized in Fig. 9.3.

The second part of the thesis explores the effectiveness of paclitaxel in the treatment of disease in the ND4 mice. As the experiments in part one were underway, use of paclitaxel to treat the ND4 mice was initiated. The rationale for the use of paclitaxel was primarily its antiproliferative properties although it has been reported to have other actions as well. These actions will be discussed later. Since a large increase in astrocyte numbers was reported previously (Mastronardi et al., 1993) and confirmed in this thesis with additional astrocyte counts and glial fibrillary protein (GFAP) quantitation, an antiproliferative drug was the agent of choice.

These animal studies provided the data required for the approval of a clinical trial (phase I/II) of paclitaxel in chronic progressive multiple sclerosis which began at St. Michael's Hospital in August 1998.
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Chapter 1: Introduction

The focus of this thesis is on demyelination and the mechanism resulting in disease. As will be presented in later chapters, a spontaneously demyelinating animal model was employed to elucidate the biochemical reactions involved in demyelination and also to test the effectiveness of a drug for the treatment of demyelinating disease. These studies were undertaken with the intention of application towards the human demyelinating disease Multiple Sclerosis (MS). In order to better understand the disease process in MS, a review of myelin and its components will be discussed first. This will be followed by a discussion on the pathology and etiology MS, animal models of demyelinating disease, and various treatments for MS.

Myelin is a multilamellar membrane sheath formed by membranous extensions from Schwann cells in the peripheral nervous system (PNS) (Gerens, 1954) and oligodendrocytes in the central nervous system (CNS) (Peters, 1960a; Peters, 1960b; Bunge et al., 1961; Bunge et al., 1962). A unique membrane, myelin ensheaths and insulates large portions of axons forming an internode and facilitates the high velocity conduction of nerve impulses between unmyelinated gaps known as the nodes of Ranvier (Fig. 1.1)(Ranvier, 1871; Tourneux and LeGoff, 1875). This process is known as saltatory conduction. Each Schwann cell myelinates a portion of a single axon whereas an oligodendrocyte can ensheathe up to 50 different axons (Peters, 1964; Bunge and Glass, 1965; Hirano, 1968; Peters and Proskauer, 1969).

Myelin is characterized under electron microscopy (EM) by a repeating pattern of electron dense lines formed by the juxtaposition of the membrane surfaces of the oligodendrocyte. The apposition of the cytosolic surfaces of the membrane forms the major dense line (MDL) while the apposition of the extracellular surfaces forms what is
Figure 1.1: Diagram showing an oligodendrocyte connected by its processes to several internodal myelin segments. (Adapted from Morell and Norton, 1980).
Figure 1.2: Specialized features of myelin. Electron micrographs showing features of myelin. a) Cross section of CNS myelin internode with the major dense line (MDL) and the intraperiod line marked by arrows. (Adapted from Raine, 1977) b) Schmidt-Lanterman incisure from PNS myelin. (Adapted from Kirshner and Blaurock, 1992) c) Radial component (arrows) extend through the myelin internode produced by an oligodendrocyte (O) in this section from a normal mouse (From Nagara and Suzuki, 1982). d) Node of Ranvier (*) showing the alignment of paranodal loops (arrows) around an axon (A) (Adapted from Raine, 1977).
actually a space termed the intraperiod line (IPL) (Fig. 1.2a) (Robertson, 1955). Using X-ray diffraction, the periodicity of successive MDLs has been measured to be 153-159 Å in the CNS which is smaller than the periodicity in the PNS (171-182Å) (Worthington and Blaurock, 1968).

In addition to compact myelin, the internodes contain other specializations such as the Schmidt-Lanterman incisures (Schmidt, 1874; Zawerthal, 1874; Lanterman, 1877). More prominent in the PNS, these cytoplasmic tracks run through the myelin from the periaxonal region to the glial cell soma and are incorporated into the internode in a spiraling cone-like fashion appearing in a V-shape in longitudinal sections of myelin (Fig. 1.2b). The radial component is another specialization which runs perpendicular to the nerve fiber axis and radially through the internode (Fig. 1.2c) (Peters, 1961; Honjin et al., 1963). Seen typically in the CNS, this is a network of interlamellar junctions each containing cytoplasmic and extracellular domains. At the periphery of the internode adjacent to the node of Ranvier, each layer of membrane opens up to form pockets of cytoplasm known as paranodal loops (Fig. 1.2d) (Robertson, 1957; Uzman and Norgueira-Graf, 1957). Tight junctions attach neighboring loops to each other and to the axon (axonglial junction). In the PNS, tight junctions attach paranodal loops of adjacent internodes to form glial-glial junctions (Dermeitzel, 1974a; Dermeitzel, 1974b; Reale et al., 1975).

A) Myelin Composition in CNS

I) Lipids

Myelin is elaborated from the plasma membrane yet its composition is different from that of most cell membranes. Unlike other cell membranes, myelin has a higher lipid to protein ratio (70% lipid and 30% protein by dry weight, Table 1.1). This higher lipid content gives myelin a more buoyant property which enables its isolation by gradient fractionation in a relatively purified fraction (Norton and Poduslo, 1973a).
The lipid composition of myelin in CNS differs from most other membranes in that it contains a lower proportion of phospholipids and a higher proportion of cholesterol and galactolipids. The major lipids in myelin are cholesterol, cerebrosides, and ethanolamine phosphoglyerceride (Table 1.1). Less abundant lipids include plasmalogens, phosphatidylcholine, and sphingomyelin. Expressed in mole ratios, the major lipids vary between a ratio of 4:3:2 and 4:4:2 of cholesterol, phospholipid, and galactolipid (Norton and Cammer, 1984). Although there are no myelin specific lipids, cerebroside can be classified as a "myelin typical" lipid since its relative amount is proportional to the amount of myelin (Norton and Poduslo, 1973b). The composition between species are relatively similar with the exception of the rat which has less sphingomyelin.

**Table 1.1: Composition of adult rat CNS myelin.** aTotal protein and lipid figures are expressed as a percentage of the dry weight; all others are expressed as a percentage of the total lipid weight. bPlasmalogens are primarily ethanolamine phosphoglycerides. cFrom Norton and Cammer, 1984.

<table>
<thead>
<tr>
<th>Weight (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Protein</strong></td>
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</tr>
<tr>
<td><strong>Total Lipid</strong></td>
<td>70.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>27.3</td>
</tr>
<tr>
<td>Total Galactolipid</td>
<td>31.5</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>23.7</td>
</tr>
<tr>
<td>Sulfatides</td>
<td>7.1</td>
</tr>
<tr>
<td>Total Phospholipids</td>
<td>44.0</td>
</tr>
<tr>
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</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>11.3</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>7.0</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>1.2</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>3.2</td>
</tr>
<tr>
<td>Plasmalogens(\text{b})</td>
<td>14.1</td>
</tr>
</tbody>
</table>
II) Proteins

The proteins of myelin are dominated by two families of proteins. The proteolipid proteins (proteolipid protein (PLP) and DM20) account for ~50% of total myelin proteins while myelin basic proteins account for ~35%. Less abundant proteins include 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and oligodendrocyte myelin glycoprotein (OMgp).

*Proteolipid Proteins*

The family of myelin proteolipid proteins include two major members, proteolipid protein (PLP) (Folch and Lees, 1951) or lipophilin (Gagnon et al., 1971) and DM20 (Agrawal et al., 1972), both of which are derived from the PLP gene. PLP has a molecular mass of 30 kDa whereas DM20, an alternative splice product has a molecular mass of 26 kDa (Nave et al., 1987; Simons et al., 1987). Together, they account for ~50% of total myelin protein (Norton and Poduslo, 1973b). Both proteins are integral membrane proteins that are thought to play a role in myelin compaction (Boison and Stoffel, 1994; Klugmann et al., 1997). However, increased expression of DM20 in early stages of growth suggest that DM20 has another role possibly in cell maturation (Ikenaka et al., 1992; Timsit et al., 1992; Knapp, 1996). These proteins will be described in greater detail in a later section.

*Myelin Basic Protein (MBP)*

Myelin basic protein (MBP) like myelin proteolipid proteins is also a heterogeneous family of proteins derived from one single gene. Size heterogeneity ranging from 21.5 kDa to 14 kDa in the mouse and charge heterogeneity arise from alternative splicing (Campagnoni and Macklin, 1988) and different post-translational modifications (Moscarello, 1990a) respectively. These peripheral membrane proteins have been localized
to the MDL although the charge isomer component 8 (C8), the citrullinated MBP, has been detected in the IPL by immunogold labelling (McLaurin et al., 1993). The major isoform in human has a molecular weight of 18.5 kDa whereas in rodents the 14 kDa is the major isoform. These positively charged proteins are thought to be essential for the formation and the compaction of myelin (Roach et al., 1983) perhaps through interactions with myelin lipids (Boggs et al., 1981) and proteins (PLP) (Braun, 1984; Wood and Moscarello, 1989). Other studies also suggest that MBP may be related to low molecular weight G-proteins and perhaps be actively involved in intracellular signal transduction (Boulias, 1993). Related to this function, Hardy et al. found that MBP was localized to the nucleus among other areas within an oligodendrocyte (Hardy et al., 1996). The mechanism of MBP transport into the nucleus was found to occur in a regulated and ATP-dependent manner. These authors speculated that MBP may play a specific function within the nucleus, such as regulation of gene expression or RNA processing. A more in depth discussion of MBP will follow in a later section.

**2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase)**

The Wolfgram proteins were first described as a set of high molecular weight proteins termed Wolfgram components 1 and 2 (W1 and W2) (Wolfgram, 1966). While both W1 and W2 have since been shown to be doublets, the components of W1 have been identified as different isoforms of 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase) (Drummond and Dean, 1980; Sprinkle et al., 1980; Sprinkle et al., 1983).

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase), the first myelin-specific enzyme identified, is an early marker for oligodendrocytes (Braun et al., 1988) and accounts for ~4% of total myelin proteins (Drummond et al., 1962; Kurihara and Tsukada, 1967). It has been localized by immunohistochemical methods to the oligodendrocyte membrane, paranodes, paraxonal region (Kurihara and Tsukada, 1967; Braun et al., 1988;
Sprinkle, 1989), and also the MDL (Tsukada and Kurihara, 1992). Isolated as an insoluble precipitate in chloroform: methanol, CNPase resolves in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) into a doublet with an apparent molecular mass of 46 kDa and 48 kDa in humans. They form the W1a and W1b bands of Wolfgram component 1 (W1) (Drummond and Dean, 1980; Sprinkle et al., 1980; Sprinkle et al., 1983). Its insolubility and association with the membrane may be due to three sequences of 4 hydrophobic amino acids in the N-terminus (Tsukada and Kurihara, 1992) and to isoprenylation of the C-terminus (Braun et al., 1991; DeAngelis and Braun, 1994; DeAngelis and Braun, 1996a). Although the mechanism of CNPase action in myelin remains unknown, recent studies using a transgenic mouse overexpressing CNPase suggests that this enzyme helps to target MBP to compact myelin and more importantly, participate in regulating the distribution of submembranous actin filaments (Gravel et al., 1996; DeAngelis and Braun, 1996b; Gravel et al., 1997; Yin et al., 1997).

Myelin Associated Glycoprotein (MAG)

Myelin associated glycoprotein (MAG) is a transmembrane glycoprotein of the immunoglobulin (Ig) superfamily. More abundant in the CNS (~1% of total protein) than in the PNS (~0.1%) (Quarles et al., 1973), the glycosylated protein has a Mr of 100kDa due to the presence of carbohydrates. The core protein contains five extracellular Ig-like domains, a transmembrane segment, and a cytoplasmic domain. Alternative splicing at the RNA level and phosphorylation modify the cytoplasmic domain giving rise to two polypeptides with molecular masses of 72 kDa and 67 kDa (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987) that are preferentially expressed during development. The larger isoform of MAG is the major form in early stages of myelination while the smaller isoform is more abundant in mature myelin. Both have been localized using immunogold labeling and immunocytochemistry to the periaxonal region in CNS myelin; however, the larger
isoform has also been found in multivesicular bodies within developing oligodendrocytes (Sternberger et al., 1979; Trapp and Quarles, 1982; Martini and Schachner, 1986; Trapp et al., 1989b). Thirty percent of all MAG in the CNS contains asparagine-linked carbohydrate (Quarles et al., 1983).

The function of MAG in myelin remains elusive though several roles have been suggested. Structural similarities between MAG and the adhesion molecules L1, J1, and N-CAM and the presence of both the RGD sequence (an amino acid motif responsible for adhesion) (Lai et al., 1987) and the HNK-1 carbohydrate epitope (an important determinant of adhesion molecules) (Poltorak et al., 1987) suggest that MAG is a cell adhesion molecule in myelin. This has been demonstrated by the inhibition of axon-oligodendrocyte and oligodendrocyte-oligodendrocyte interactions using Fab fragments specific to MAG (Poltorak et al., 1987). A recent study demonstrating increased Fyn tyrosine kinase activity during peak myelination and the binding to and enhancement of Fyn kinase activity by MAG, suggests that MAG also has a role in myelination (Umemori et al., 1994). Furthermore, MAG has been found to interact with a phospholipase C (PLCγ) (Jaramillo et al., 1994) and to colocalize with F-actin and spectrin (Trapp et al., 1989b). These data suggest that MAG is involved in secondary messenger signaling and cell motility, perhaps during myelin formation. In addition, MAG has also been shown to inhibit neurite growth in vitro (McKerracher et al., 1994; Mukhopadhyay et al., 1994). In spite of these findings, transgenic mice deficient in MAG survive and develop normally, forming normal compact myelin (Li et al., 1994). Based on the assumption that MAG plays a major role in myelin formation, the objective of this experiment was to provide insight into the function of MAG in the central nervous system. Ironically, the results indicate that MAG is not necessary for normal myelin formation and maintenance. Although MAG may have a role in cell adhesion, intracellular signaling, and in directing neurite growth, the normal development of MAG deficient mice indicates that other molecules are able to compensate in its absence.
Myelin Oligodendrocyte Glycoprotein (MOG)

Myelin oligodendrocyte glycoprotein (MOG), an oligodendrocyte-specific protein, was first identified in 1986 (Lebar et al., 1986) and later localized to the outermost surface of myelin (Brunner et al., 1989). Like MAG, MOG is also a member of the immunoglobulin (Ig) superfamily; however, MOG contains one Ig-like domain and two transmembrane domains which associate with the membrane by forming a hair-pin loop (Gardinier et al., 1992; Pham-Dinh et al., 1994; Hilton et al., 1995; Gardinier et al., 1997). Resolving into a doublet at 26 kDa and 28 kDa in SDS-PAGE, MOG has a molecular mass of 25 kDa upon deglycosylation. The localization of MOG to the outer surface of myelin suggest that it may mediate extracellular cell signaling (Quarles et al., 1997) or act as an autoimmune antigen for demyelinating disease (Schluesener et al., 1987; Lassmann et al., 1988; Xiao et al., 1991; Amor et al., 1994). In lesions from acute MS and marmoset allergic encephalomyelitis, antibodies specific to MOG were found to be bound to disintegrating myelin (Genain et al., 1999). This suggested that MOG antibodies may mediated membrane targeted damage in demyelinating disease. However, this was challenged by a more recent study which demonstrated increased anti-MOG antibodies in the CSF and plasma from both MS patients and patients with other non-demyelinating neurological diseases (Kami et al., 1999). These investigators concluded that anti-MOG antibodies were not diagnostic for MS and suggested that in MS MOG has a defensive role against immune-mediated damage after myelin breakdown by opsonizing myelin debris to facilitate phagocytosis by macrophages. The function and importance of MOG in MS is still under investigation.
*Oligodendrocyte-Myelin glycoprotein (OMgp)*

Oligodendrocyte-myelin glycoprotein (OMgp) is an oligodendrocyte-specific protein with a molecular mass of 120 kDa (Mikol and Stefansson, 1988). Found in human white matter and on the surface of cultured ovine oligodendrocytes, OMgp is highly glycosylated with both N-linked and O-linked oligosaccharides which account for ~30 kDa of the protein (Mikol and Stefansson, 1988). This protein contains four distinct domains: a short cysteine-rich sequence in the N-terminus, a segment containing 7 1/2 tandem leucine-rich regions, a serine/theronine-rich region, and a hydrophobic C-terminus (Mikol et al., 1990). It is through this C-terminus and its association with a phosphatidylinositol which binds OMgp to the membrane. The remainder of the molecule, which resembles several adhesion molecules, likely plays a role in cell adhesion. This is implied by a subpopulation of OMgps containing the HNK-1 carbohydrate (Mikol et al., 1990). OMgp has also been shown to bind peanut agglutinin through O-linked oligosaccharides within the serine/theronine-rich domain (Mikol and Stefansson, 1988; Mikol et al., 1990).

**B. Myelin Proteolipid Protein**

When Folch and Lees first isolated the protein components from the chloroform: methanol soluble fraction of bovine whole brain homogenate, they found that like other lipoproteins, these components contained both lipid and protein (Folch and Lees, 1951). However, unlike lipoproteins, the components had a higher lipid to protein ratio, so they named them proteolipids to distinguish them from lipoproteins. Folch and Lees also realized that these proteolipids behaved differently from other lipoproteins in that they were insoluble in water but soluble in chloroform: methanol and that they were trypsin resistant. This property made the myelin proteolipids very difficult to work with and it was not until
over thirty years after their isolation that the primary sequence was reported and later verified by cDNA cloning.

Today, we realize that the myelin proteolipids isolated by Folch and Lees are actually a family of proteins derived from the same gene. The name proteolipid protein (PLP) is commonly reserved for the protein product from the full length gene (PLP gene) although others have referred to it as lipophilin or N₂ in the past (Gagnon et al., 1971; Boggs and Moscarello, 1978; Gow, 1997). DM20, the other product of the PLP gene, arises from an alternative splice site (Agrawal et al., 1972; Macklin et al., 1987; Nave et al., 1987; Simons et al., 1987). Not restricted to the CNS, PLP and DM20 have also been found in Schwann cells but not in the myelin in PNS (Puckett et al., 1987; Kamholz et al., 1992). In spite of our cumulative knowledge of PLP/DM20 thus far, its three-dimensional structure and its function in myelin remains to be elucidated.

I) Primary Structure of PLP

The primary structure of PLP was first fully determined for bovine brain PLP by proteolytic and chemical digestion followed by sequence analysis of the peptides (Fig. 1.3) (Lees et al., 1983; Stoffel et al., 1983). By piecing the peptide fragments together, it was determined that PLP consisted of 276 amino acids, the majority of which are hydrophobic or apolar (>60%). Its sequence has since been confirmed using cDNA clones from different species (Milner et al., 1985; Macklin et al., 1987). The primary structure of PLP was found to be highly conserved between species with absolute conservation (100% sequence homology) between mouse, rat, and human. Two positions differ between rat and bovine at amino acids, Phe¹⁸⁸→Ala¹⁸⁸ and Ser¹⁹⁸→Thr¹⁹⁸ while human and rabbit PLP differ only at one amino acid, Ser¹⁹⁸→Thr¹⁹⁸. (Tosic et al., 1994). Presently, PLP sequences have also been deduced for dog (Nadon et al., 1990) and chicken (Schliess and Stoffel, 1991), and a partial sequence for frog (Schliess and Stoffel, 1991).
<table>
<thead>
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<th>No.</th>
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<tr>
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<td>Gly-Leu-Leu-Glu-Cys*-Cys*-Ala-Arg-Cys*-Leu-Val-Gly-Ala</td>
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<td>14</td>
<td>Pro-Phe-Ala-Ser-Leu-Val-Ala-Thr-Gly-Leu-Cys-Phe-Phe-Gly</td>
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<tr>
<td>42</td>
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</tr>
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<td>56</td>
<td>Asp-Tyr-Glu-Tyr-Leu-Ile-Asn-Val-Ile-His-Ala-Phe-Gln-Tyr-Val</td>
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<td>265</td>
<td>Ala-Val-Leu-Leu-Leu-Met-Gly-Arg-Gly-Thr-Lys-Phe</td>
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</table>

**Figure 1.3**: Amino acid sequence of mouse/human PLP. Primary sequence of PLP showing the sites of modification: *, naturally palmitoylated cysteine residues; †, cysteine residues in disulfide bonds. Sequence missing from DM20 protein is underlined.
Residues in italics are the changes resulting from a frame shift mutation in the Jimpy mouse. Amino acid changes in bovine (B) and rabbit (f) sequence.

II) Post-translational Modifications of PLP

A highly basic protein with an isoelectric point of ~9.2 (Draper et al., 1978), PLP contains two disulfide bonds and covalently bound fatty acids as the only post-translational modifications. PLP was the first protein found to contain covalently bound fatty acids (Gagnon et al., 1971; Stoffyn and Folch-Pi, 1971). Approximately 2-3 moles of fatty acid is bound per mole of protein which, in addition to the abundance of hydrophobic amino acids give the protein its extreme hydrophobic property. The majority of covalently bound fatty acids are composed of palmitate (53-60%), stearate (8-10%) and oleate (25-35%) (Stoffyn and Folch-Pi, 1971). These fatty acids were first thought to be covalently bound through oxyester bonds (Stoffyn and Folch-Pi, 1971; Cockle et al., 1980) with Thr^{198} identified as a potential site of acylation (Cockle et al., 1980; Stoffel et al., 1983).

Ross and Braun however suggested that the fatty acids were attached through thioester bonds because of their sensitivity to sodium borohydride (Ross and Braun, 1988). This was confirmed using peptide fragments of PLP that were reduced and carboxymethylated using hydroxyamine at neutral pH and (^{14}C) iodoacetamide (Bizzozero and Good, 1990). Utilizing a fluorescent-labeling technique in which cysteines residues were derivatized by N-(iodoacetylaminoethyl)-5-naphthylamine-1sulfonic acid (I-AEDANS) directly or after reduction, the sites of fatty acylation were found to be at cysteine residues 5, 6, 9, 108, 138, and 140. Two disulfide bonds were found to occur between Cys^{200}-Cys^{219} and Cys^{183}-Cys^{227} (Fig. 1.3) (Weimbs and Stoffel, 1992).

Acylation of PLP has been extensively characterized using both in vitro and in vivo systems. Acylation of PLP has been suggested to occur close to or within the myelin
membrane and not at the site of synthesis. This was shown by incubating brain tissue slices with blockers of protein synthesis and transport (Townsend et al., 1982; Bizzozero et al., 1983). In another experiment in which brain slices were incubated with $^{3}$H-palmitic acid, Townsend et al. found that lipid was added to PLP in myelin and myelin-like membranes (Townsend et al., 1982). Since the specific activity of PLP was found to be greater in the myelin-like membrane than in myelin, the authors suggested that a pool of PLP existed in the myelin-like membrane from which pre-acylated PLP is transferred to myelin (Townsend et al., 1982). Once in myelin, PLP can be deacylated and acylated again. The extent of acylation depends on the pool of acyl donors (fatty acyl-CoA) and not on the pool of unacylated protein (Bizzozero et al., 1986b). In fact, the turnover rate of the bound fatty acids has been measured to be 3-4 days in vivo (Bizzozero and Good, 1991a). The sites of acylation are also highly specific for palmitoyl-CoA with a Km value of 41 μM. This preference can change with changes in the fatty acid pool, for example, an increase in covalently bound long chain fatty acids has been observed in PLP isolated from patients suffering from adrenal leukodystrophy (ALD), a disease characterized by the accumulation of long chain fatty acids (Bizzozero et al., 1991b).

PLP was thought to undergo autoacylation since acylation was inhibited by SDS and heat but not by cerulenin, an inhibitor of acyl transferases (Bizzozero et al., 1987). In their study, the investigators incubated PLP with $^{3}$H palmitoyl-CoA for 20 min after which the protein was precipitated with acetone. However, the conclusion made from this experiment must be carefully evaluated since the protein was not washed to extract the remaining fatty acyl-CoA. Although acetone may remove some non-covalently bound fatty acyl Co-A, PLP is extremely hydrophobic and can readily form tight associations with these reactants. The possibility that PLP may have retained some of the fatty acyl-CoA group in this assay makes their conclusion questionable since the increase in radioactivity observed in the recovered PLP could have been due to the retention of non-covalently
bound $^3$H palmitoyl-CoA. Deacylation of PLP was thought to be due to the activity of a PLP specific myelin-associated fatty acylesterase found in rat brain myelin (Bizzozero et al., 1992). This enzyme has been shown to deacylate PLP in vitro (Bizzozero et al., 1992).

Ever since PLP was first found to be palmitoylated, the list of palmitoylated proteins has grown. This includes proteins such as G-proteins, G-protein coupled receptors and several members of the ras protein family (Milligan et al., 1995; Morello and Bouvier, 1996). Palmitoylation of these proteins has been demonstrated to affect their interactions with lipid and with other proteins, for example, palmitoylation of rhodopsin aids the association of the C-terminal tail with the membrane and with G-proteins (O'Brien and Zatz, 1984; Ovchinnikov et al., 1988). Similarly, palmitoyl groups on PLP and DM20 may also be important for their activity and interactions. Using ultraviolet and intrinsic fluorescence spectroscopy to study the exposure of Trp and Tyr residues in PLP and deacylated PLP, Bizzozero and Lees found that these residues were more exposed in an aqueous environment in the deacylated protein than in the acylated protein (Bizzozero and Lees, 1986a). Although no structural analysis was employed, these authors suggested that the presence of fatty acids may alter the folding of the protein (Bizzozero and Lees, 1986a). However, unless the function of PLP is elucidated, the role of fatty acid groups on PLP remains speculative.

III) PLP Gene Structure

The PLP gene, first described in the human and later in the mouse (Deihl et al., 1986; Macklin et al., 1987), has a high degree of identity within both non-coding regions and coding regions between the two species (Fig. 1.4). Of the 831 nucleotides coding for the 277 amino acids of PLP, only 25 bp differ between the human and mouse and 11 bp differ between mouse and rat. All changes are completely conserved at the amino acid level.
The PLP gene is localized to the X-chromosome in the region q13-q22 (Willard and Riordan, 1985; Deihl et al., 1986) and is comprised of approximately 17kb containing seven exons. Exon 1 is largely made up of the 5'-untranslated sequence which has 94% identity between mouse and human and a small coding sequence at its 3'-end for a methionine and the first base pair in the codon for glycine, the first amino acid in PLP. As predicted from cDNA sequencing, the N-terminal methionine is cleaved off and the protein does not contain a signal peptide (Milner et al., 1985; Deihl et al., 1986). Exon 1 lies 7 kb upstream from the rest of the exons in the PLP gene (Fig. 1.4).

According to the currently accepted model of PLP (Weimbs and Stoffel, 1992), each of the remaining exons (2-7) encodes a hydrophobic domain and the adjacent hydrophilic sequence of the protein, with the exception of exons 5 and 6. Exon 5 encodes an extracellular loop containing two disulfide bonds while exon 6 encodes the first half of the C-terminal transmembrane domain. The remainder is encoded within exon 7. The rest of exon 7 contains 3'-untranslated region.

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**Figure 1.4:** Splicing pattern of the PLP gene in normal mouse. (a) A single mouse PLP gene is 17 kb in length and contains 7 exons. Alternative splicing of exon 3 produces two mRNA products encoding (b) PLP (full length transcript) and (c) DM20 (transcript lacking exon 3b). *(Adapted from Nave et al, 1987).*
Since only 200 nucleotides of the human exon 7 were sequenced, the 3'-untranslated region has only been compared between mouse and rat. This region has 90% identity between mouse and rat and contains three polyadenylation sites beginning at nucleotides 1,481, 2,310, and 3,176 (Fig. 1.4). These are consistent with three different size mRNA found in mouse brain at approximately 1.5-1.6 kb, 2.4 kb, and 3.2 kb in length. The major PLP mRNA present in rat, mouse, human, and bovine is 3.2 kb in length (Milner et al., 1985; Naismith et al., 1985; Gardinier et al., 1986; Kronquist et al., 1987). The minor isoforms differ between species with the exception of bovine which only contains the 3.2 kb isoform. The 2.4 and 1.6 kb products are both found in rat and mouse while a 2.2kb mRNA is found in human (Kronquist et al., 1987).

The transcription start sites for the PLP mRNA from mouse, rat, baboon, and human begin at approximately 146-160 bp upstream from the methionine codon. Comparing the placement of the TATA box, the 5'-untranslated region appears to be shorter in the human than in the mouse by ~40 bp (Deihl et al., 1986). Nevertheless, this site lies within an 80 nucleotide segment that has 100% identity between mouse and human. The region immediately upstream to the transcription start site (+1 to -225) is sufficient for maximal activity (Fig. 1.5a) (Berndt et al., 1992; Cambi and Kamholz, 1994) and contains several key cis-acting DNA sequences for maximal tissue-specific expression of PLP in cultured glial cells (Fig 1.5b) (Berndt et al., 1992). Using these sites as probes, a glia-specific protein related to the zinc-finger family of transcription factors, myelin transcription factor-1 (MyTi-1), was cloned (Kim and Hudson, 1992). Myt 1 is thought to play a distinct role in the development of the nervous system (Kim et al., 1997) since it is expressed in oligodendrocytes in early stages of development (Armstrong et al., 1995) and promotes neuronal differentiation in Xenopus (Bellefroid et al., 1996).
Figure 1.5: The PLP promoter region from mouse. The PLP promoter contains regions that enhance (black bars) and regions that suppress (white bars) transcription. Within the region proximal to the transcription start site are five sequences found to be involved in the binding of transcription factors for maximal transcriptional activity.

IV) DM20 Protein

Alternative splicing of the PLP gene produces a smaller isoform termed DM20 protein (Fig. 1.4). DM20 was first identified in the CNS as a 20.5 kDa protein on SDS-PAGE (Agrawal et al., 1972). Migrating slightly ahead of PLP, DM20 was thought to be an alternatively folded form of PLP since antibodies raised against PLP reacted with DM20. Ironically, implications that PLP and DM20 were different proteins resulted from later immunoreactive studies using an antibody raised against the amino acids 117-129 in PLP (Trifilieff et al., 1986). In this study, the antibody reacted with PLP but not with DM20 suggesting that DM20 is missing the amino acid sequence 117-129. This deletion was confirmed and characterized only after the PLP gene was cloned. Several groups demonstrated that exon 3 contains an alternative donor splice site which when used deletes
105 nucleotides resulting in the removal of amino acids 116-150. This deletion remains in frame and produces mRNA encoding the 241 residue DM20 protein (Macklin et al., 1987; Nave et al., 1987; Simons et al., 1987).

V) Developmental Expression of PLP and DM20 Protein

The developmental expression of PLP follows a caudal to rostral direction in the developing brain (Macklin et al., 1983; LeVine et al., 1990; Schindler et al., 1990). Macklin et al. examined the developmental expression of PLP, DM20 and MBP in rat myelin from various areas of the central nervous system and demonstrated by immunoblot analysis that these proteins accumulated first in the spinal cord at 2 days followed by the lower brain stem at 4 days, the cerebellum at 4-6 days and then the upper brain stem by 10 days (Macklin et al., 1983). Although PLP is found in low amounts before myelin is formed, a sharp increase in its expression is marked by the onset of myelination (Nussbaum and Mandel, 1973; Macklin et al., 1983; Gardinier and Macklin, 1988; LeVine et al., 1990). In the mouse, the period of active myelination lasts from post-natal day 14 (P14) to day 27 (P27). PLP levels peak by day 22 (P22) (Campagnoni and Hunkeler, 1980; Gardinier and Macklin, 1988) and drop thereafter decreasing gradually by approximately 1/5 by P44 (Campagnoni and Hunkeler, 1980).

DM20 protein was thought to have the same pattern of expression as PLP. With the discovery that DM20 is a different protein from PLP, the developmental expression of PLP and DM20 has since been shown to be different. DM20 protein is expressed in abundance during early development while PLP is more abundant in adult animals. Interestingly, this was first hinted at, but not measured by Agrawal et al.. In their study, these investigators found that DM20 occurred in the developing brain in addition to brain samples from more mature animals (Agrawal et al., 1972). It was not until 1987 that DM20 protein levels were shown to precede and to exceed PLP levels in early
development. Using immunoblots of human fetal spinal cords at different ages, Kronquist et. al. saw that DM20 but not PLP was present by 18 weeks post-conception and the relative amount of DM20 was more abundant than PLP at 21 weeks post-conception (Kronquist et al., 1987). DM20 was found to be the main product in fetal bovine brain, while PLP did not appear until 4 weeks afterwards (Schindler et al., 1990). In mice, DM20 was detected on immunoblots as early as P3 while PLP does not appear until P5 (Gardinier and Macklin, 1988). In another study, the ratio of DM20 to PLP in fetal bovine brain was 12 times higher than the ratio measured in both adult bovine and rat brain (0.5-0.8 and 0.7-0.8 respectively) (VanDorsselaer et al., 1987). The sharp increase in synthesis of PLP corresponds with the onset of myelin formation.

Although PLP levels are detected in low amounts several days after birth in rodents, its accumulation and assembly into myelin begins with myelin formation (LeVine et al., 1990). Western blots of brain homogenate from mice and rats at different ages demonstrated that PLP levels accumulate rapidly after 11 days and exceed DM20 levels by P14 (Gardinier and Macklin, 1988; LeVine et al., 1990). At adult ages (> 2 months), DM20 levels are approximately 1/4 the levels of PLP. Early developmental stages (premyelinating) are marked by higher DM20 protein levels while more developed (post-myelinating) stages are marked by higher PLP protein levels (Knapp, 1996).

VI) Developmental Expression of PLP and DM20 mRNA

Like the developmental expression of PLP and DM20 protein, the mRNAs for the two proteins also follow a caudal to rostral direction in the brain with temporally shifted peak periods of expression. In the mouse, DM20 mRNA was detected as early as embryonic day 10 (E10) by in situ hybridization and embryonic day 11 (E11) by reverse transcriptase polymerase chain reaction (RT-PCR) (Ikenaka et al., 1992; Timsit et al., 1992). Peak DM20 mRNA levels were found to occur at E14 although no protein was
detected at this age (Ikenaka et al., 1992). PLP mRNA was detected in low amounts only after birth within the oligodendrocyte cell body (Verity and Campagnoni, 1988; Shiota et al., 1989; Timsit et al., 1992). Peak PLP mRNA expression corresponded with peak protein levels at P21 and was maintained until P27 (Sorg et al., 1987). These levels decreased thereafter to 20-30% of peak levels at older ages (Shiota et al., 1991).

The signal responsible for the increased synthesis of PLP and decreased synthesis of DM20 protein remains elusive. In a study by Levine et al., it was suggested that the developmental regulation of PLP and DM20 occurs at a post-transcriptional or post-splicing stage (LeVine et al., 1990). Their conclusions were based on the finding that PLP mRNA was consistently higher than DM20 mRNA and that the changes in protein levels were not reflected by changes in the amount of mRNA. In light of the work in embryonic mice by Timsit et al. (Timsit et al., 1992) and Ikenaka et al. (Ikenaka et al., 1992) which took place after Levine’s experiments, this conclusion may have to be re-evaluated since all samples used were from post-natal rats.

Alternatively, Cook et al. (Cook et al., 1992) concluded from their studies on PLP mRNA in cultured oligodendrocytes that there is strong transcriptional control over PLP expression during development. They found that the rate of transcription of the PLP gene paralleled the mRNA steady state while the half-life of PLP mRNA was measured to be relatively short (25 hours, in vitro) (Cook et al., 1992) compared to the protein (t1/2>28 days (Townsend et al., 1982; Bizzozero et al., 1983). In a recent experiment, Mathisen et al. measured a shorter half-life for PLP mRNA of 5 hours using brain slices incubated in media (Mathisen et al., 1997). Since Cook et al. measured PLP mRNA half-life in cultured oligodendrocyte, the discrepancy between these two findings may be due to the conditions under which their cells were maintained. The system used by Cook et al. may have extended the stability of the mRNA in the cultured cells thus giving a longer half-life for PLP mRNA. Although these results provide insight towards the regulation of the PLP
gene, these findings do not address how PLP synthesis is increased relative to DM20 since they did not specifically examine DM20 mRNA in their study. Further studies are needed to identify the stage at which the change from DM20 to PLP synthesis occurs.

VII) Secondary Structure of PLP

The specific orientation of PLP in myelin has been difficult to obtain because of the hydrophobic nature of PLP. What is known is that PLP is a multimembrane spanning protein with both transmembrane and extramembranous domains. Labelling of PLP with the hydrophobic photoactivatable probe, 3-(trifluoromethyl)-3-([125]I)iodophenyl) diazirine suggests the presence of three transmembrane domains (Kahan and Moscarello, 1985). Recognition by PLP specific antibodies and digestion of membrane bound PLP with proteases implicates the presence of both transmembrane and extracellular domains (Lin and Lees, 1985). Several models have been proposed based on hydropathy profiles, immunoreactivity in a lipid environment, susceptibility to tryptic digestion, and the position of covalently bound fatty acids. These are discussed below.

Laursen's Model:

The first model proposed was based entirely on the primary structure and predicted hydrophobic regions (Laursen et al., 1984). In this model, the protein was predicted to contain three membrane spanning domains and two cis-membrane domains that enter and exit the bilayer on the same side (Fig. 1.6a). These two domains were thought to function in myelin formation and maintenance of stability by inserting into adjacent bilayers on the extracellular surfaces. At the cytosolic surface, a highly charged segment containing the sequence spliced out of DM20 (E1 in Fig. 1.6a) was believed to be arranged in β-sheet conformation. This portion was thought to stabilize myelin by interacting with MBP or lipids in the opposite membrane surface. The arrangement of the E1 loop on the
cytoplasmic side of the membrane was supported by the reactivity to cultured oligodendrocyte by an antibody specific to this region only after the cells have been permeabilized (Trifilieff et al., 1986). Similarly, immunoreactivity of the C-terminus after permeabilization of cultured mouse oligodendrocytes also supported its placement on the cytosolic side of the membrane (Konola et al., 1992). Since both studies examined the position of these segments of PLP in the cell membrane of oligodendrocytes, the orientation of PLP in myelin can only be inferred from their results.

**Stoffel’s Model**

A similar multi-membrane spanning model was proposed at the same time based on analysis of peptides released from PLP in bovine myelin by tryptic digestion (Fig. 1.6b) (Stoffel et al., 1984). Although the N- and C- terminus were positioned in the same manner as in Laursen’s model, the orientation of domains was vastly different. In Stoffel’s model, the three membrane spanning domains were inverted while the two cis-membrane domains both resided closer towards the C-terminus. More importantly, the extramembranous loops were arranged on opposite sides. In particular, the highly charged loop (loop B in Fig. 1.6b) resided on the extracellular side. Stoffel et al. assumed that no digestion occurred on the cytosolic side since MBP was detected at the correct molecular weight on SDS-PAGE in myelin samples treated with trypsin. Although intact MBP was present in these samples, Popot et al. noted that the intensity of staining was reduced (Popot et al., 1991). They suggest that during the preparation of myelin, osmotic shocking may breakup the membrane sufficiently to allow for some tryptic activity to occur on the cytosolic side of myelin membranes. This may explain the occurrence of the highly charged loop (loop B in Fig. 1.6b) in Stoffel’s results and its placement in this model.
Figure 1.6. Proposed models for the orientation of PLP in a lipid bilayer.

A) Laursen model. (Laursen et al., 1984)
B) Stoffel model. (Stoffel et al., 1984)
C) Hudson model. (Hudson et al., 1989)
D) Popot model. (Popot et al., 1991)
E) Weimbs model. (Weimbs and Stoffel, 1992)
F) Greer model (Greer et al., 1996)
Hudson's Model

Using an oligodendrocyte culture system, Hudson et al. proposed a third model based on antibody reactivity to non-permeabilized and permeabilized cells (Fig. 1.6c) (Hudson et al., 1989). This model contained four α-helical domains two of which were embedded in and spanned the lipid bilayer. The remaining helices and the rest of the molecule resided outside of the membrane on the extracellular surface. Both the N- and the C-terminus were also on the extracellular side. Although mapping of the protein in the membrane was carefully done using PLP specific antibodies, the positioning of most of the molecule on the extracytosolic side of the membrane seems unlikely considering the high content of apolar residues in PLP and previous labelling experiments which suggest that a larger portion of the protein exists within the bilayer and on the cytosolic side (Kahan and Moscarello, 1985; Lin and Lees, 1985; Lin et al., 1986; Trifilieff et al., 1986). Their conclusions may have also been skewed by non-specific binding of immunoglobulins to oligodendrocytes (Lubetzki et al., 1986) and PLP antibodies to astrocytes (Hartman et al., 1982).

Popot's Model

Maintaining the theory that a high proportion of PLP resides within the membrane, Popot et al. proposed a fourth model based on the hydropathy profile of PLP and on the modeling of other membrane proteins (Fig. 1.6d) (Popot et al., 1991). In this model, the cis-membrane domains proposed in both Laursen’s model and Stoffel’s model were eliminated giving PLP four membrane spanning domains. After comparing the charge distribution of the residues comprising the first membrane spanning helix in PLP with the charge distribution in helices from other membrane proteins, the N-terminus of PLP was placed in the cytosolic side of the membrane. Accordingly, both the highly charged loop
between transmembrane domains B and C (Fig. 1.6d) and the C-terminal end of PLP were positioned on the cytosolic side which corresponded to labeling studies in oligodendrocyte cell membrane (Lin et al., 1986; Trifilieff et al., 1986; Konola et al., 1992).

**Weimbs's Model**

Based on the location of fatty acylated cysteine residues in PLP, Weimbs et al. proposed yet another model (Fig. 1.6e) (Weimbs and Stoffel, 1992). Since the fatty acid donors (fatty acyl-CoA) (Ross and Braun, 1988; Bizzozero and Good, 1991a) resided on the cytoplasmic side of the membrane and since these acyl groups have a high rate of turnover (Bizzozero et al., 1992) meaning that the acylesterase must be readily accessible, these investigators concluded that the modified cysteine residues must also lie on the cytosolic side. This model agreed best with Popot’s model and is currently the most accepted of all five models.

**Greer's model**

Greer et al. examined the orientation of PLP in cultured oligodendrocytes using antibodies specific to different PLP peptides (Greer et al., 1996). From their studied, these investigators proposed a model (Fig. 1.6g) similar to the one described by Weimbs et al.. However, in addition to the four transmembrane domains, Greer et al. suggested that the segment spanning amino acids 206 to 215 in PLP was inserted into the membrane bilayer since no immunolabelling was observed with two different antibodies specific to this region of PLP. This was in agreement with earlier findings (Laursen et al., 1984) (Fig 1.6 A). Since these experiments were performed in a cultured oligodendrocytes system, Greer's model supports the orientation of PLP proposed by Weimbs et al. within a cell membrane with a slight modification of the second extracellular domain.
To date the actual structure of PLP within a lipid environment remains unsolved. At best, models that fit the predictions or the experimental data have been proposed. However, agreement between models based on theoretical predictions and experimental findings must concur to give the most accurate structure. The only model that best satisfies this is Greer’s model. This model is based largely on immunolabelling studies using cultured oligodendrocytes and is supported by data on the sites of fatty acylation (Weimbs and Stoffel, 1992) and also by labelling studies on PLP reconstituted in lipid vesicles (Lin et al., 1986; Trifilieff et al., 1986; Konola et al., 1992). Stoffel’s model whose orientation contradicts that of Weimbs model and Greer’s model is also supported by Laursen’s theoretical model and by their own data obtained from tryptic digestion of PLP in myelin. However, this orientation is inconsistent with the labelling studies (Lin et al., 1986; Trifilieff et al., 1986; Konola et al., 1992). Although they assumed that no digestion occurred on the cytoplasmic surface of myelin bilayers, the process of osmotic shocking during the preparation of myelin may break up and expose cytoplasmic surfaces of the bilayer to trypsin activity. Thus the orientation of PLP proposed by both Laursen’s model and Stoffel’s model remains questionable and are not widely accepted. The model of PLP proposed by Hudson et al. contrasts with all the other models in that most of the molecule resides outside of the lipid bilayer. This is not a favorable orientation for PLP since it is a highly hydrophobic protein and as such does not fit any predicted structures. Furthermore, this orientation is inconsistent with both the PLP/vesicle studies (Lin et al., 1986; Trifilieff et al., 1986; Konola et al., 1992) and the labelling studies by Kahan and Moscarello which suggests that PLP contains at least three transmembrane domains (Kahan and Moscarello, 1985). The conclusions made by Hudson et al. may have been influenced by non-specific binding of antibodies to oligodendrocytes and astrocytes in their cultured cell. Thus, Greer’s model for PLP is considered the most accurate of all the other models proposed. With an accurate model for PLP, insight into the function of the protein can be obtained.
Chapter 1

VIII) Function of PLP and DM20

PLP has long been thought to be important both in myelin formation and compaction. This is implied by its abundance and its peak expression during the onset of myelination. However, this commonly held theory has been challenged by the findings in PLP knock-out mice (Boison and Stoffel, 1994; Klugmann et al., 1997). These mice develop normally and elaborate myelin of normal appearance (Rosenbluth et al., 1996; Klugmann et al., 1997). However, closer examination, showed that the periodicity was slightly reduced from 11.1 nm in wild-type myelin to 10.3 nm in myelin from knock-out mice (Rosenbluth et al., 1996). Also, the myelin was found to be extremely susceptible to disruption. In certain areas, the MDL and the IPL were indistinguishable. In these areas, the thickness of the IPL was reduced while in other areas pockets of separations were observed (Rosenbluth et al., 1996). This study implied that PLP plays a role in stabilizing compact myelin structure but is not essential for its formation. The authors argue that the absence of PLP results in a lack of firm intermembrane bonding which results in structural instability. These bonds may be formed through interactions with lipids and other myelin proteins such as MBP (Wood and Moscarello, 1989). In a recent study, PLP reconstituted into phospholipid vesicles was demonstrated to mediate vesicle membrane rolling (Palaniyar et al., 1998). This result further suggests that PLP contains adhesive properties with lipids.

Impairment of motor control in PLP deficient mice was also reported by several groups. Using the PLP deficient mice prepared by Klugman et al., Griffiths et al. demonstrated a progressive decrease in motor control with age using the ‘rotarod’ test (Griffiths et al., 1998). Using the same method, Boisson and Stoffel also observed an impairment in motor coordination in their PLP deficient mice (Boison and Stoffel, 1994). Boisson and Stoffel also found that the axonal conductance was reduced by half to which
they attributed the motor defects. The reduction in axonal conductance was postulated by Griffiths et al. to be the result of axonal swelling and degeneration found in small caliber axons in PLP deficient mice (Griffiths et al., 1998). They attributed this defect to the loss of local oligodendroglial support. This was thought to be mediated through the "channel-like" properties of PLP (Griffiths et al., 1998) since in vitro studies using isolated myelin vesicles and PLP reconstituted into lipid vesicles have demonstrated that PLP can function as an ion channel (Helnyck et al., 1983; deCozar et al., 1987; Robertson et al., 1989) in a Na\(^+\) ion and protein concentration dependent manner (Ting-Beall et al., 1979). Griffith et al. argue that a deficiency in this function in oligodendrocytes compromises axonal integrity which results in the disruption of axonal transport and accumulation of membranous dense bodies followed by axonal swelling and axonal degeneration.

Whether this defect suggested by Griffiths et al. can be attributed directly to the loss of PLP is debatable. In both PLP deficient mouse lines, the animals survived to at least 16 months of age and were able to reproduce normally. In their study, Griffiths et al. found that the loss of motor control was significant only after 16 months of age (Griffiths et al., 1998), well after the peak period of myelin formation which occurs between 14 to 21 days after birth in mice. If one takes into consideration that the normal life-span of a mouse is approximately two years and that the myelin from these transgenic mice was deficient in 50% of the total proteins present in normal myelin, the significance of this impairment is minor at best. Alternatively, the changes in motor control in the PLP deficient mice as measured by Griffiths et al. could have been due to a loss of balance arising from the onset of old age or a gain of weight with age. The observed axonal expansion in these animals was also questionable. In a study using optic nerves from mice, Sanchez et al. demonstrated that axons expanded upon ensheathment by normal oligodendrocytes (Sanchez et al., 1996). In their PLP deficient mice, Boisson and Stoffel did not observe any signs of axonal swelling (Boison and Stoffel, 1994). Both these
studies contradict the conclusion made by Griffith et al. Furthermore, the axonal swellings found by Griffith et al. do not seem to be significant since only a small percentage of cells (\(\sim 11\%\)), only small caliber axons, and only restricted areas of the brain were affected. Swelling was also localized only to the paranodal regions while the whole length of the axon was unaffected. Since these transgenic mice were able to survive and reproduce normally in the absence of PLP, the role played by PLP in oligodendrocytes as suggested by Griffiths et al. is not convincing.

The results from the studies on PLP deficient mice indicate that the role played by PLP in myelin may not be as important as was once thought. Although PLP seems to be required for maintaining the structural integrity of the myelin sheath, it was not essential for myelin formation and compaction. Like MAG, the absence of PLP was not detrimental to the normal survival of the these animals.

Unlike PLP, DM20 protein may play a role outside of myelin. DM20 has been found to be related to a larger family of proteins that includes the DM genes in sharks (Kitagawa et al., 1993; Sinoway et al., 1994) and the M6 protein (proteins that are localized to the leading edge of neuronal growth cones (Yan et al., 1993). Since DM20 expression peaks at a premyelinating stage, it may play a role in cell differentiation (Knapp, 1996).

Expression of PLP and DM20 protein in animals containing naturally occurring mutants of PLP also suggest that DM20 has a role in cell development and survival. Naturally occurring mutations of the PLP gene usually results in a lethal phenotype, for example, mutations of the PLP gene in both the Jimpy (Jp) mouse (Phillips, 1954) and the Jimpy myelin-synthesis deficient (Jp\(^{msd}\)) mouse (Meier and MacPike, 1970) (Table 1.2) produces a dysmyelinating phenotype accompanied by premature death (Meier and MacPike, 1970; Billings-Gagliardi et al., 1980). Several groups have proposed that
mutation of the primary structure of PLP results in a misfolded protein or abnormal interactions with other proteins in the endoplasmic reticulum (Roussel et al., 1987; Gow et al., 1994; Jung et al., 1996). These investigators suggest that the abnormal proteins may perturb all cellular membrane trafficking and result in oligodendrocyte cell death and hypomyelination in these mutant animals. The complete absence of PLP in the PLP-deficient mice may have a smaller ranging effect which may explain the less severe phenotype compared with animals with missence mutations (Boison and Stoffel, 1994; Klugmann et al., 1997).

Both Jp and Jp<sup>msd</sup> mice have a shortened life span that lasts approximately one month. Although a different protein is produced in the Jp mice (Fig. 1.3) (Hudson et al., 1986; Nave et al., 1986), these mutants have extremely low amounts of both PLP and DM20 protein (Kerner and Carson, 1984; Yanigasawa and Quarles, 1986; Gardinier and Macklin, 1988; Duncan et al., 1989; Benjamins et al., 1994). However, in PLP mutant mice in which DM20 protein is produced in normal or higher amounts, oligodendrocytes tend to survive and form myelin. This is observed in the Jimpy rumpshaker mouse (Jp<sup>rsh</sup>) (Schneider et al., 1992) and the paralytic tremor rabbit (pt) (Tosic et al., 1993) (Table 1.2).
which have a milder phenotype compared with Jp mice and Jp<sup>msd</sup> mice. Both Jp<sub>rsh</sub> and pt mice survive to adulthood and are able to reproduce successfully (Griffiths et al., 1990; Tosic et al., 1993). Although both PLP and DM20 are affected by these mutations, the major PLP gene product in these animals has been found to be DM20 while PLP levels remain low (Mitchell et al., 1992; Tosic et al., 1993; Tosic et al., 1994). Since mutations in the PLP gene are usually lethal except in cases where DM20 is produced, DM20 likely plays a role in oligodendrocyte survival and development.

C) Myelin Basic Protein (MBP)

In the early 1960's, a protein in the water soluble basic protein fractions from CNS myelin was found to induce experimental allergic encephalomyelitis (EAE) (Einstein et al., 1962; Kibler et al., 1964), a disease in animals characterized by focal areas of demyelination. This protein was later isolated and identified in acid and salt extracts of delipidated bovine brain homogenates and is now referred to as myelin basic protein (MBP) (Martenson and LeBaron, 1966; Nakao et al., 1966). To date, MBP is the most extensively characterized protein of the central nervous system myelin. It comprises approximately 35% of total myelin proteins in the CNS myelin and 15-20% in the PNS myelin (Lees and Brostoff, 1984). It is localized to the MDL in the myelin of both CNS and PNS (Omlin et al., 1982). However, MBP is thought to be involved in myelin compaction only in the CNS (Rosenbluth, 1980a; Rosenbluth, 1980b).

1) The MBP Gene and Protein Isoforms

The MBP gene in both human and mouse consists of 7 exons which codes for a 21.5 kDa protein (Fig. 1.7) (deFerra et al., 1985; Takahashi et al., 1985; Kamholz et al.,
1986; Roth et al., 1986; Newman et al., 1987; Roth et al., 1987). Alternative splicing of
the gene transcript produces mRNA coding for 20.2 kDa, 18.5 kDa, 17.2 kDa, 17 kDa,
and 14 kDa proteins in the mouse (Barbarese et al., 1977; Greenfield et al., 1982; Newman
et al., 1987) and 20 kDa, 18.5 kDa, and 17.2 kDa proteins in human (Kamholz et al.,
1986; Roth et al., 1986; Roth et al., 1987). These isoforms are the result of splicing of the
full length transcript at exons 2, 5, and 6 (Fig. 1.7). The expression of each isoform is
developmentally regulated (Barbarese et al., 1978; Carson et al., 1983). In the adult
mouse, the 18.5kDa and 14kDa isoforms are the major isoforms while in adult humans, the
18.5kDa isoform is the predominant form. Other isoforms that are expressed at embryonic
stages of development have been identified.

Recent studies demonstrated that the MBP gene itself is part of the larger Golli-
MBP gene (gene expressed in oligodendrocyte lineage-MBP)(Fig. 1.8). The transcripts
from this gene were found not only to be expressed in embryonic stages of development in
the brain but also in the spinal cord and other non-neural cells or tissue (thymus, spleen,
human B-cells, and macrophage cell lines) in both mouse and human (Campagnoni et al.,
1993; Pribyl et al., 1993). In another study at least 18 new isoforms of MBP mRNA were
found in embryonic mouse brain. These novel isoforms were separated into three groups
based on their developmental profiles. One group of mRNAs which lacked exon 5 had
peak levels of expression at E15 (Nakajima et al., 1993). Alternatively, high molecular
weight MBP-related proteins were expressed in both a human oligodendrocyte cell line
(MO3-13) and in white matter (Ursell et al., 1995).

II) MBP gene transcription

Several binding sites for different transcription factors may be important for the
regulation of MBP expression. In vitro studies have shown that the MBP promoter region
between nucleotides -54 and -253 upstream of the transcriptional start site was sufficient
Figure 1.7: Alternative splicing of MBP RNA gives rise to five different mRNA species in the mouse and four mRNA species in the human. (Adapted from Campagnoni and Macklin, 1988)

for brain-specific MBP transcription while the region between nucleotides -130 and -111 was necessary for brain-specific transcription (Tamura et al., 1989). Located within this region is a binding site for the transcription factor nuclear factor 1 (NF-1) (Tamura et al., 1988; Miura et al., 1989) which has been implicated for brain-specific MBP expression.
Chapter 1

Golli-MBP Gene
Golli-MBP exons

MBP exons

Transcription

Golli-MBP hnRNA

Alternative Splicing

BG21 mRNA
(31 kDa protein)

J37 mRNA
(35 kDa protein)

TP8 mRNA
(7.5 kDa protein)

Figure 1.8: The Golli-MBP gene carrying the classical 7 exon MBP gene products. (Adapted from Campagnoni, 1993).

(Aoyama et al., 1990). The region proximal to the transcription start site between nucleotides -14 and -50 has been shown to suppress MBP transcription (Devine-Beach et al., 1990). A 38 - 41 kDa protein isolated from mouse brain homogenate (myelin gene expression factor-2 (Myef-2)) was found to bind specifically to this region and to repress MBP expression in transient tranfection assays. Its expression in mouse brain was coordinated with the onset of MBP expression (Haas et al., 1995). In addition to these
transcription factors, the MBP promoter also contains binding sites for other transcription factors such as the activator protein-1 (AP-1) transcription factor. These site are located distal to the start site; however, further studies are required to determine the role played by these transcription factor in MBP expression. Like the PLP gene, transcription of the MBP gene involves several different transcriptional elements acting in both positive and negative capacities.

III) Primary Structure of MBP

The primary structure of MBP was first elucidated in human and bovine species (Carnegie, 1971; Eylar et al., 1971) and later in mouse (Dunkley and Carnegie, 1974). MBP occurs in various isoforms ranging from 21.5 kDa to 18.5 kDa (Kamholz et al., 1986; Roth et al., 1986; Roth et al., 1987). An additional 14 kDa isoform was found in rodents (deFerra et al., 1985; Takahashi et al., 1985). Each isoform arises from alternative splicing of the MBP gene transcripts which has been discussed in the previous section.

Of the different isoforms, the best characterized and most abundant isoform in normal adult human and bovine is the 18.5 kDa isoform (Fig. 1.9). This sequence consists of 170 amino acids in human (Carnegie, 1971) and 169 amino acids in bovine (Brostoff and Eylar, 1971) and is approximately 90% conserved between different species (bovine, human, rat, mouse, chicken, rabbit, and guinea pig) (Stoner, 1990). The primary sequence consists of a large portion of basic amino acids (24%) including 12 lysyl and 19 arginyl residues. The number of histidines (10) are unusually high while acidic amino acids make up approximately 6.0% of the residues. This gives MBP a basic isoelectric point (pI ≥ 10.5) (Carnegie, 1971). MBP also contains high proportions of prolyl residues. Of interest is a triproline sequence near the middle of the molecule which is thought to form a hairpin loop (Eylar et al., 1971) and a potential binding site for MAP kinase (Yon et al.,
Figure 1.9: Amino acid sequence of human MBP. Primary sequence of human MBP showing the arginine residues deiminated to citrulline residues (in bold) in MBP-C8. Changes found in the bovine sequence are compared to the human sequence and are marked below the corresponding position: underlined residues, deleted in bovine sequence; italicized residues, substituted by residue below in bovine sequence; Ser76 is replaced by Ala-Gln-Gly.
1996)(Fig. 1.10). Structurally, no disulfide bonds exist in MBP since it does not have any cysteinylic residues.

IV) Post-translational Modifications of MBP

MBP has the potential to be highly modified since at least 20 sites have been shown to be modified by at least 8 different types of post-translational modification (Fig. 1.10). Most of these modifications seem to be restricted to the N- and C-termini while the middle
portion (residues 55-94) contains relatively fewer modifications. The modifications can be grouped into those that do not alter the charge of the protein and those that change the charge of the protein. Interestingly, the change in charge is unidirectional towards a loss of positive charge. It is this group of modifications that gives rise to the charge microheterogeneity of MBP.

MBP Charge Isomers

In addition to the size heterogeneity, MBP can be separated into different charge isomers by cation-exchange chromatography (CM-52) at pH 10.6 (Deibler and Martenson, 1973; Chou et al., 1976) (Fig. 1.11a). These charge isomers can also be fractionated on alkaline-urea gels (Fig. 1.11b). The charge microheterogeneity of MBP is attributed to one or a combination of post-translational modifications resulting in a change in the net positive charge. These include phosphorylation, deamidation, C-terminal arginine loss, methionine oxidation and deimination (Deibler et al., 1975; Chou et al., 1976; Chou et al., 1977; Martenson et al., 1983; Cheifetz et al., 1984; Wood and Moscarello, 1989). Using the nomenclature proposed by Deibler and Martenson (Deibler and Martenson, 1973) to describe the different charge isomers, component 1 (C1) refers to the least modified and most cationic isomer, C2 refers to the isomer that is less positive than C1 by one charge, C3 by two positive charges, and so on in reverse order of elution from the CM-52 columns at pH 10.6 (Fig. 1.11a). The least positively charged isomer is found in the void volume and is referred to as C8. Each isoform of MBP contains its own set of charge isomers (Palma et al., 1997). The strength of the interaction of each isomer with anionic lipids depends upon the number of positive charges. The implications of the changes in the number of charges on the structure and function of MBP in a bilayer will be discussed in greater detail in a subsequent section. Of importance to the pathogenesis of demyelinating disease is the deimination of MBP in the formation of MBP-C8. The following sections
Fraction No.

A

Figure 1.11: Separation of human 18.5 kDa MBP into charge isomers. MBP can be separated into isomers based on charge. Each isomer differs from the adjacent isomer by one positive charge. The least cationic isomer which comes off the column in the void volume is termed C8 which consists of two peaks (C8a and C8b). The most positively charged and least modified isomer (C1) elutes off the column last. A) CM52 column profile of human MBP; B) Alkaline-urea tube gels of unfractionated MBP (SM) and isolated MBP charge isomers. (From T. Miani) —— Absorbance (260nm) ——— Conductivity (milli S)
will focus on deimination of MBP while the other modifications such as phosphorylation will not be discussed since these have not been characterized in MBP-C8 isolated from diseased myelin.

**MBP-C8**

MBP component 8 (C8) is a collection of proteins which arise from the deimination of arginines to citrullines (Wood and Moscarello, 1989). These proteins are collected within the void volume of a CM-52 column in two peaks (C8-A and C8-B) (Fig. 1.11a). Further purification by HPLC resolved a high $M_r$ component and a low $M_r$ component for each A and B fractions in the 17- to 18 kDa range (Boulias et al., 1995). Masses of 18 558.08 and 17 266.03 Da were reported for the high and low $M_r$ components of C8-B respectively. C8-A was not analyzed as yet. These components were shown to be related to MBP by Western blot, partial sequencing, and by mass spectrometry (Boulias et al., 1995). One of the C8 components from human 18.5kDa MBP was found to contain 6 citrulline at positions 25, 31, 122, 130, 159, and 170 (Wood and Moscarello, 1989). Subsequent amino acid analysis of the other C8 components demonstrated a gain of citrullines with a loss of arginine residues as well (Boulias et al., 1995). Another form of MBP C8 isolated from the myelin of a patient suffering from fulminating Multiple Sclerosis (MS) (Marburg’s disease) has been characterized and shown to have 18 of its 19 arginyl residues converted into citrullinyl residues (Wood et al., 1996). Thus the citrullinated forms of MBP can be classified based on the number of citrulline present, for example, MBP-Cit 0 (or MBP-C1), MBP-Cit 6 (the naturally occurring MBP-C8 with 6 citrullinyl residues), and MBP-Cit 18 (Marburg-type C8 with 18 citrullinyl residues). The implications of increased citrulline in MBP will be discussed in a following section. Other post-translational modifications such as phosphorylation may also be present in C8 but have not been characterized.
Properties of C8

The citrullinated form of MBP (MBP-Cit \(_6\)) differs from the other charge isomers in its properties, its expression during development, and its localization in myelin. Using an antibody specific to C8 (recognizing only citrulline), this protein was localized to the IPL in myelin by immunogold labeling while C1 was localized primarily to the MDL using an antibody specific to residues 130-137 of MBP (McLaurin et al., 1993). C8 was found in human infant brains between 5 days to 15 months of age (Moscarello et al., 1994), and in young normal mice (Fannon and Moscarello, 1991). By comparing the relative ratio of C8 to C1, C8 proteins were more abundantly expressed in early myelinating stages than at adult ages in both human and mouse (Moscarello et al., 1994; Mastronardi et al., 1996b). Thus high C8 levels relative to C1 acts as a marker for an early stage in oligodendrocyte development. Its interactions also differ compared with other isomers. Due to the loss of positive charges, C8 cannot induce aggregation of anionic lipid vesicles. However, it can induce PLP dependent aggregation of phosphatidyl choline (PC) vesicles (Wood and Moscarello, 1989). Since PC is found mainly on the outer leaflet of the bilayer (Braun, 1984), the interaction between C8 and PLP in PC vesicle may explain its localization at the IPL. The mechanism by which protein bound arginine is converted to citrulline involves the enzyme peptidylarginine deiminase (PAD) (Ec 3.5.3.15).

Peptidylarginine Deiminase (PAD)

Citrulline was first detected in proteins from vertebrate epidermis, the medulla of hair fibers, and porcupine quills (Harding and Rogers, 1971). A fraction of myelin proteins was also shown to contain citrulline (Finch et al., 1971). These proteins were later shown to have the properties of MBP-Cit \(_6\), the citrullinated form of MBP (Wood and Moscarello, 1989). Since the citrullines in MBP-Cit \(_6\) are nonrandomly positioned (2 at the
N-terminus, 4 at the C-terminus) and only MBP contains citrulline in myelin, this suggests that the conversion of arginine into citrulline occurs through an enzymatic reaction. The only enzymatic mechanism by which arginine in MBP is converted to citrulline is by the activity of peptidylarginine deiminase (PAD) in brain (Lamensa and Moscarello, 1993).

![Figure 1.12: Reaction of PAD with benzoylarginine ethyl ester (BAEE) forms benzoylcitrulline ethyl ester (BCEE).](image)

Peptidylarginine deiminase (PAD) (EC 3.5.3.15) is an enzyme that modifies arginine into citrulline within proteins (Fig. 1.12). PAD has been found in various tissues (Takahara et al., 1986; Takahara et al., 1987; Takahara et al., 1989) including hair follicles (Harding and Rogers, 1971), epidermis (Kubilus et al., 1980; Sugawara et al., 1982), skeletal muscle (Sugawara et al., 1982; Hosokawa et al., 1983), and brain (Kubilus and Baden, 1983). Three isoforms of PAD have been found each expressed in different tissues (Terakawa et al., 1991). The isoform found in brain is PAD type II (Takahara et al., 1989). Both mRNA and protein for PAD type II have been localized to spinal cord (Watanabe and Senshu, 1989; Akiyama et al., 1990) and more recently the protein was detected in the periaxonal region of myelin by immunogold labeling (Pritzker, 1996). In a study using partially purified PAD from bovine brain, MBP was shown to be an excellent
substrate in vitro (Lamensa and Moscarello, 1993), since 17 of the 19 arginyl residues in MBP were deiminated into citrulline. In another study, an increase in PAD activity was found in whole brain homogenate of DM20 overexpressing transgenic mice at three months of age (Mastronardi et al., 1996b). MBP isolated from these mice had increased levels of C8 relative to C1 (Mastronardi et al., 1996b). The results from the in vitro study and the findings in the DM20 transgenic mice implies that MBP is a substrate for PAD activity in the brain and that C8 is produced by the deimination of MBP in vivo. High amounts of C8 and increased PAD activity may play a role in destabilizing myelin as will be discussed later in reference to demyelinating disease. Further studies however are needed to correlate the developmental expression of C8 with the developmental expression and the activity PAD in brain.

V) Expression of MBP

The expression of MBP is highly co-ordinated with PLP expression and with the onset of myelination (Campagnoni and Hunkeler, 1980; Verity and Campagnoni, 1988). Detectable in mice as early as P12 in oligodendrocyte cell processes and by P14 in myelin, MBP levels rise sharply thereafter and peak by P18-20, several days before peak PLP expression (Campagnoni and Hunkeler, 1980; Gardinier et al., 1986; Verity and Campagnoni, 1988; Shiota et al., 1989). After P20, the levels of MBP drop sharply to adult levels (Gardinier et al., 1986). MBP mRNA is detected as early as E12 in mice using RT-PCR (Nakajima et al., 1993). The steady state of MBP mRNA at postnatal stages parallel the appearance of the protein suggesting strong transcriptional regulation (Verity and Campagnoni, 1988; Shiota et al., 1989). While total MBP gene transcript levels peaked at P20, each isoform displays differential expression patterns.

In a study of the developmental expression of MBP isoforms in mouse, Barbarese et. al. found that each isoform had a different rate of accumulation (Barbarese et al., 1978).
The ratio of the isoforms, in order of size (21.5kDa, 18.5kDa, 17kDa, 14kDa) was 1:5:2:10 between 10 and 30 days (the 17.2kDa isoform was not considered). Over the next 30 days, this ratio was 1:10:3.5:35. The accumulation of the different isoforms was correlated with mRNA levels (Carson et al., 1983). Similar results were found in humans (Kamholz et al., 1988). These findings demonstrated that the 21.5kDa and 17kDa isoforms are more abundant earlier in development while the 18.5kDa and 14kDa isoforms are dominant in more mature myelin. Since both the 18.5kDa and 14kDa proteins lack amino acids encoded by exon 2, MBP isoforms containing exon 2 may play a role during active myelination.

VI) Secondary Structure of MBP

Early studies on MBP in aqueous solution using circular dichroism (CD) (Eylar and Thompson, 1969; Anthony and Moscarello, 1971; Block et al., 1973; Epand et al., 1974), nuclear magnetic resonance spectroscopy (NMR) (Mendez et al., 1982), and Fourier transform infrared spectroscopy (FTIR) (Surewicz et al., 1987) demonstrated that MBP had no α-helical or β-sheet structure. However, using other methods, other investigators suggested that MBP contained some folded structure. This was implied by the loss of fluorescence enhancement of MBP and fluorescence energies of tyrosyl and tryptophanyl residues in MBP upon addition of a denaturing agent (Jones and Epand, 1980; Randall and Zand, 1985). Using low-angle x-ray scattering data, Epand et. al. proposed that MBP had a rod-like structure with dimensions of 15 X 150 Å (Epand et al., 1974). In a lipid environment, MBP exhibits α-helical structure in varying degrees depending on the lipid used and the lipid to MBP ratio (Anthony and Moscarello, 1971; Keinry and Smith, 1981; Gow et al., 1990). The conflicting results from these studies indicate that the secondary structure of MBP remains under debate. The variability of MBP structural determinations may be due to changes induce by extraction and purification procedures and may also be
influenced by the microheterogeneity of MBP. As such the finding of any secondary structures or lack thereof for MBP may only be predictive. Although these studies do not provide a definite secondary structure for MBP, MBP structure appears to be susceptible to changes in environment.

Figure 1.13: Topologies of arrangement of β-strands in the Stoner (a) (Stoner, 1984) and Martenson (b) (Martenson, 1981) models of MBP.

Based on three predictive algorithms for secondary structure, two models of MBP (Fig. 1.13) consisting largely of β-sheets were also proposed (Martenson, 1981; Stoner, 1984). In a separate experiment this was demonstrated by CD which showed that the charge isomers C1 to C4 had 10-20% β-sheet conformation (Ramwani et al., 1989; Deibler et al., 1990). Due to its microheterogeneity, MBP crystals have been impossible to obtain for structure determination. In a recent study, a 3 dimensional model of MBP was
predicted for human MBP (C1) in association with a lipid monolayer using two dimensional data obtained from electron microscopy (Beniac et al., 1997; Ridsdale et al., 1997). Based on the β-sheet conformation proposed by Stoner and Martenson, but not on the arrangement of the sheet, this model was proposed to be “C”-shape. At a resolution of 2.8 nm, this model had the following dimensions: outer radius, 5.5 nm; inner radius, 3 nm; height, 4.7 nm; overall circumference, 15 nm. Since the MDL is only ~1.7 nm thick, these dimensions imply that MBP would penetrate deeply into the lipid bilayers. The authors speculated that the particle they analysed were MBP trimers thus explaining the large dimensions of their model. Whether this relationship exist within myelin still remains to be resolved. Deimination of MBP alters this structure resulting in a more expanded shape (Beniac, personal communication). Since C8 has been localized to the IPL (McLaurin et al., 1993), the wider spacing of the IPL (2.5 nm) may accommodate the larger molecule. Although these models are highly hypothetical and the resolution of this technique is comparatively low, these findings suggest that the charge microheterogeneity can potentially alter the conformation of MBP and as a result its function and interactions within myelin.

VII) MBP Lipid Interactions

MBP has been shown to interact with lipid in a bilayer in two ways; through electrostatic interactions with anionic lipids and through hydrophobic interaction by the insertion of hydrophobic segments of the protein into the bilayer. It is thought that the electrostatic interactions occur first followed by penetration of hydrophobic segments into the bilayer (Boggs and Moscarello, 1978).

Hydrophobic interactions between MBP and a lipid bilayer involve apolar residues and fatty acid chains within the bilayer. The apolar residues in MBP are thought to insert into the bilayer and interact with fatty acid chains. Alternatively, the lipid chains may be
exposed to these residues by a distortion in the bilayer (Boggs and Moscarello, 1978). Portions of MBP inserted into the lipid layer were protected from tryptic digestion (London and Vossenberg, 1973) and were shown to occur at the N- and the C- termini of MBP (Jones and Rumsby, 1977; Boggs et al., 1981; Menon et al., 1990). Although it may not act as a permanent anchor, N-terminal acylation of MBP may also aid in this interaction (Moscarello et al., 1992). However, a stronger bond between MBP and lipid is formed by the electrostatic interactions.

The electrostatic interaction between MBP and anionic or acidic lipids depends on the phase state of the lipid, the type of lipid used, and also the charge on MBP (the charge isomer). Smith and Braun found that MBP/lipid interactions were inhibited by divalent cations (Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$) and by treating phospholipid vesicles with phospholipase C (Smith and Braun, 1988). Conversely, they found that upon treating phospholipids with phospholipase D, which generated phosphatidic acid (PA), MBP was not released. They concluded that MBP interacted best with anionic lipids. This interaction was shown to occur at the N- and C-terminus of MBP in a charge dependent manner in two different studies. Boggs et al. cleaved MBP at the Trp residue to obtain two fragments of different size and charge. Upon competition for binding to anionic lipids using Mn$^{2+}$, the authors found that the amount of lipid protected by these fragments corresponded with the number of positive charges present (Boggs et al., 1981). Using MBP fragments generated by the same method, Sankaram et al. demonstrated that the moles of dimyristol-phosphatidylglycerol bound to each peptide also corresponded with the number of positive charges (Sankaram et al., 1989). The strength of the interaction between MBP and lipids was dependent on the cationic nature of MBP (Cheifetz and Moscarello, 1985a; Moscarello et al., 1986).

Post-translational modification of MBP has been shown to decrease MBP-lipid interactions by reducing the positive charge on MBP and by altering its structure. Wood
and Moscarello demonstrated that the effectiveness of phosphatidylcholine:phosphatidylserine (PC:PS (92.2\%:7.8\%)) vesicle aggregation was dependent on the number of positive charges on MBP. They found that C1 was most effective in vesicle aggregation assays. MBP-C2 was less effective than C1 while C3 was less effective than C2. MBP-C8 was the least effective of all isomers studied (Wood and Moscarello, 1989). As mentioned in the previous section, C8 was found to have an expanded structure compared to C1 (Beniac, personal communication). This change in structure may also alter the interaction of C8 with membrane lipids or proteins. In studies by Cheifetz et al., it was found that phosphorylation of C1 and C2 decreased the aggregation of PC:PS (92.2\%:7.8\%) vesicles and the release of a water-soluble spin-label (tempocholine chloride) from within PS:PC vesicles (1:11.5, w/w) (Cheifetz and Moscarello, 1985a; Cheifetz et al., 1985b). These results were attributed to the loss of positive charge on C1 and C2 which reduced their interaction with anionic lipids and their ability to penetrate the bilayer. In another study, Costentino et al. demonstrated that with increased length of alkyl chains attached at the N-terminus of the N-terminal peptide 1-21 from MBP, the degree of random structure and the extent of phosphorylation was also increased (Costentino et al., 1994). Not only does N-terminal acylation reduce the cationicity of the peptide by promoting phosphorylation but it also alters its structure. The results from these studies suggest that the post-translational modification of MBP plays an important role in regulating MBP-lipid interaction and by extension the stability of myelin. This is mediated through electrostatic interactions between MBP and lipids and also through changes in conformation. Large decreases in the cationicity of MBP such as an accumulation of MBP-Cit_{18} in fulminating MS results in a less stable myelin which may become more susceptible to breakdown.
**D) Multiple Sclerosis (MS)**

I) Pathology of MS

Multiple Sclerosis (MS) is a disease affecting young adults with an increased prevalence in females over males (2:1) [Vollmer and Waxman, 1991]. This disease results in a loss of axonal conductance and presents as relapsing remitting episodes in patients. Most of these patients become chronic progressive. First described in 1868 by Charcot, MS is characterized by areas of focal demyelination in the CNS [Charcot, 1869; Vollmer and Waxman, 1991]. These areas, called plaques, are featured by the local loss of myelin. Plaques in MS appear without specificity and can be found in areas ranging from the optic nerve to cerebellar white matter to the spinal cord in the CNS. Plaques also occur within grey matter from MS patients [Allen and Kirk, 1997]. However, clinical signs of MS do not always correlate with plaque formation and location since some lesions have been found to be asymptomatic. Although the axon was originally thought to be preserved within the plaques, recent findings by Trapp et al. suggested that axonal transection was a consistent feature in MS lesions [Trapp et al., 1998]. This conclusion was based on the appearance of axonal ovoids stained for non-phosphorylated neurofilaments [Trapp et al., 1998]. The authors concluded that these structures represented the terminal end of transected axons; however, they did not explain why only one ovoid was observed instead of two in close proximity as would be expected from transection. Further studies are needed to confirm their conclusion. In addition to demyelination, perivascular inflammation is often observed accompanied by lymphocytic infiltration and astrocyte and macrophage hypertrophy. These features become increasingly pronounced with progression of disease. Remyelination is also observed in and near plaque areas, perhaps in an attempt to recover [Prineas and Connell, 1979; Prineas et al., 1993]. Occurring alone
or in combination, clinical signs include: motor weakness in limbs, optic neuritis, paresthesia, sensory ataxias, vertigo, vomiting, seizures, and bladder dysfunction.

II) Etiology of MS

MS is the leading cause of neurological disorders in young adults with a population frequency of approximately 0.1% in developed societies (Robertson, 1955). Age of onset commonly occurs between 20 to 40 years of age. Onset occurs slightly earlier in females than in males. Results from several studies have delineated geographic boundaries for MS based on latitude. In general, incidence and prevalence increases with distance from the equator (Acheson, 1963). However, MS has a complex etiology that involves not only an environmental component but also both immunological and genetic factors. Although most studies on MS have focused on the immunological aspects of the disease, this component may be a secondary response to a primary defect. Results from recent studies aimed at identifying genetic loci susceptible to MS suggested that several loci each playing a small but important role in the pathogenesis of disease were involved (Ebers et al., 1996; Haines et al., 1996; Kuokkanen et al., 1996; Sawcer et al., 1996). Together, these loci may define a genetic profile that increases susceptibility to either environmental and immunological components which results in demyelination. The relevance of approaching demyelinating disease from a genetic viewpoint toward understanding the mechanism of disease will be presented in the following sections.

*Environmental Factors*

Environmental factors leading to MS can be divided into geographic location and viral infection. The strongest indication of geographic factors was observed in a study of the prevalence of MS in immigrants. In this study, the investigators found that environmental risks operated at or prior to the time of puberty (Kurtzke, 1965).
Immigrants acquired the environmental risk of the new region if they migrated before the age of 15 while those who migrated after the age of 15 retain the risk of their region of origin (Dean and Kurtzke, 1970). An increase in the frequency of MS in the Orkney islands off the coast of Scotland and the Faroe islands between Norway and Iceland suggest that MS involves a viral component. Of the 25 five cases of MS reported on Faroe island between 1920 and 1977, 24 had onset between 1943 and 1960. Since the increase in incidents of MS occurred after the second world war, it was suggested that occupation of these islands by foreign troops may have introduced into the population a transmissible agent that may have a causative role in MS (Kurtzke and Hyllestad, 1979). Involvement of a viral factor was also supported by the induction of demyelination in rare cases of measles (paranyxovirus), (Allen and Kirk, 1997) induction of demyelination in normal animals by viruses, and the isolation of the measles virus, parainfluenza virus and the simian virus 5 (SV5) from tissue from MS patients (terMeulen et al., 1972; Prasad et al., 1977; Mitchell et al., 1978).

A theory by which viruses are thought to induce demyelination is through the activation of an immune response. This mechanism of action, referred to as molecular mimicry, is mediated by cytotoxic T cells activated by viral peptides that resemble peptides from myelin proteins (Fujinami and Oldstone, 1989). Eight viral peptides have been found to stimulate T cell clones specific to MBP peptide 85-99. Preserved within their sequence are the relative positions of Phe\textsuperscript{91} and Lys\textsuperscript{93} found in the MBP peptide. These residues are the T cell contact sites for MBP and are thought to serve the same role in viral peptides (Wucherpfennig and Strominger, 1995). As a result, this finding suggests that the causative agent in MS may not be due to a single virus but to a group of common viruses.

If these viral epitopes are the sites that stimulate an autoimmune reaction, one wonders why demyelination in EAE cannot be induced by injection of MBP alone but must require suspensions of MBP in Freund’s adjuvant. Upon removal of the virus,
demyelination ceases unlike in MS in which disease is persistently progressive. In light of the frequency of viral infections, the incidence of demyelination is rare which implies that certain members within a population have increased genetic susceptibility to attacks on myelin. Similarly, the findings from the migration studies and the Orkney and Faroe island studies can be interpreted as a genetic predisposition to myelin breakdown. Although these findings outline environmental and viral factors in the etiology of MS, a genetic component cannot be ruled out.

Genetic Factors

Early indications of a genetic predisposition for MS were suggested by a study on the susceptibility within families which demonstrated a concordance rate of 4% for siblings and ~3% for children and parents (population frequency is 0.1%) (Sadovnick et al., 1988). Also, the concordance rate for dizygotic twins was found to be 5% compared to a 30% rate in monozygotic twins (Sadovnick et al., 1993). In spite of cultural factors or shared environment such as diet, exposure to allergens etc., frequency of MS was found to be unchanged compared with the population frequency in a study of families with non-biological and adoptive members (Ebers et al., 1995). In addition to these studies, a disparity of susceptibility was also found between different ethnic groups living within the same region (Spurkland and Vartdal, 1997). The incidence of MS in the Hutterites population of Canada and the United States was low despite the high prevalence of MS in that part of the world. Of the cases of MS among the Hutterites, the individuals affected were found to be genetically linked to common ancestors (Hader et al., 1996). These data suggest a strong genetic factor is involved. In spite of these findings, the gene for MHC class II molecules is the only gene which has been modestly associated with MS in most genetic scans.
The HLA-DR2, DQ6 alleles of the MHC class II molecules have been found to be carried by 50-70% of caucasoid MS patients (Tiwari and Terasaki, 1987). These antigen presenting molecules expressed in T cells have been shown to recognize MBP peptide 89-99 (Martin et al., 1991). However, other genes are believed to be involved in MS since the HLA-DR2, DQ6 alleles are shared among a small proportion of siblings and account for a small percentage increase in susceptibility (Riech, 1987). In a series of genetic screens for gene loci linked to MS, several groups reported that multiple loci were associated with MS (Ebers et al., 1996; Haines et al., 1996; Kuokkanen et al., 1996; Sawcer et al., 1996). No one locus was overwhelmingly linked to disease since the LOD scores were relatively low but some of the common loci were found on chromosome 3, 5, 7 and X. Although only one group was able to find linkage to HLA-DR2 loci (Haines et al., 1996), association of a loci on chromosome 6 near the HLA loci was found after subsequent screenings (Sawcer et al., 1996) and with consideration of linkage disequilibrium (Ebers et al., 1996). These results indicate that MS is a polygenic disease involving multiple genes each with weak but additive effect towards the disease.

III) MBP in MS: Another Approach to MS

A common theme in both environmental and genetic aspects involved in MS is an autoimmune response to components of the CNS. Although the immune system has a role in demyelinating disease, there is evidence to suggest that it plays a secondary role instead of a primary role. A proposal has been put forth suggesting that myelin in MS is developmentally immature and that this less developed myelin in the adult human is the defect responsible for the autoimmune response observed in demyelination (Wood and Moscarello, 1984; Moscarello et al., 1994). Some of the evidence to support this claim will be discussed in the following section.
Myelin from MS patients has been characterized and found to resemble myelin from early development in several ways. Using wide-angle X-ray diffraction to measure phase transition temperature ($T_c$) in the myelin from normal adults and patients suffering from MS, myelin from MS patients was found to have a lower $T_c$ compared with $T_c$ from normal adult myelin. The $T_c$ of myelin from MS patients was in the range of the $T_c$ of myelin from children at age 5-8 (Chia et al., 1984). This indicates that myelin from MS patients is less stable and that instability is the result of a not fully matured myelin.

Myelin from MS patients was also characterized by high levels of C8 (MBP-Cit_6) and low levels of C1 (MBP-Cit_0) (Moscarello et al., 1994). Upon isolation and fractionation of MBP from normal and diseased brain white matter, ~45% of the MBP isolated from MS patients was made up of C8 (Moscarello et al., 1994). This correlated with the percentage of C8 found in children between the ages of 2 and 4 years old. To demonstrate that the increase in C8 was not an artifact of autopsy material, the percentage of C8 was also measured in other neurological disorders such as Alzheimer’s, motor neuron disease, Huntington’s, Parkinson’s, and amyotrophic lateral sclerosis (ALS). The amount of C8 examined in each disease was similar to normal adults (~18%) (Moscarello et al., 1994). Also, the ratio of C8 to C1 in myelin from patients suffering from these diseases was comparable with the ratio in normal adult myelin (0.82±0.59) but the C8/C1 ratio in myelin from MS patients was increased (2.45±0.5) (Moscarello et al., 1994).

Within myelin, a greater density of immunogold particle labeling for C8 is observed in myelin from MS patients (Moscarello et al., 1994) which indicated that C8 occurred naturally in tissue and was not an artifact of MBP isolation. The degree of labeling resembled the labeling pattern of myelin from normal children at age 2 (Moscarello et al., 1994). In a fulminating case of MS (Marberg type), C8 (MBP-Cit_18) was found to make up 80% of total MBP while the C8/C1 ratio was 6.7, similar to the ratio found for a 15 month old infant (Wood et al., 1996). Amino acid analysis of the MBP from this patient
demonstrated that 18 of the 19 arginines were converted into citrulline. Since this conversion corresponds to a loss of 18 positive charges, the severity of disease in MS seems to correlate with the number of positive charges lost in MBP. Both the $T_C$ measurements and the profile of MBP charge isomers in myelin from MS patients suggest that this membrane may be developmentally immature. Since MBP/lipid interactions depend on the number of positive charges on MBP, an increase in C8, the least cationic isomer of MBP, suggest that the extent of lipid interaction in myelin from MS patients and correspondingly its stability may be decreased.

**Properties of a Developmentally Immature Myelin**

Since MBP is thought to function as a stabilizing component in myelin through electrostatic interactions with acidic lipids, the loss of positive charges through the formation of C8 may result in a less stable, less compact myelin. This has been demonstrated *in vitro* using unfractionated MBP isolated from MS patients. This MBP was less effective compared with MBP from normal adults in organizing PG bilayers into multilamellar structures (Brady et al., 1981) and in aggregating vesicles (Moscarello et al., 1986). Since MBP from MS patients contain high amounts of C8 and since C8 from normal adult is also ineffective in inducing formation of multilayers from PG vesicles (Brady et al., 1981) and in promoting the aggregation of PC/PS (92.2 mol%:7.8mol%) vesicles (Wood and Moscarello, 1989), the reduced effectiveness in organizing anionic lipids by MBP from MS patients could be attributed to increased amounts of C8. Also, since loose myelin is characterized by high C8 and low C1 levels (Cruz and Moscarello, 1985), myelin in MS patients appears to be in a less compact and developmentally immature state.

A less compact, developmentally immature myelin may have increased susceptibility to deterioration at adult ages. That an immature form of myelin rather than an
autoimmune response could be the causative effect of demyelination in MS was demonstrated by an extensive examination of the C8 and C1 levels in chronic and acute experimental allergic encephalomyelitis (EAE) induce by various myelin specific antigens. In all EAE animals, the C8/C1 ratio was unchanged compared with normal mice (Mastronardi et al., 1996a). These results also indicate that the increase in C8 is not the result of acute or chronic pathology in CNS. C8 may be the antigen that induces an autoimmune response in MS. Zhou et. al. demonstrated that extent of EAE induction was increased by C8 but was reduced with increased chain length of N-terminal alkyl carboxylates on the MBP peptide 1-21 (Zhou et al., 1993). Upon isolation of C8 from MS patients, the pattern of N-terminal acylation was found to be predominantly C2 and C4 alkyl carboxylates (ie. short fatty acids) (Moscarello et al., 1994). Thus C8 found in MS patients is a potentially strong encephalitogenic antigen. In addition, an autoimmune response to citrullinated proteins is not restricted to MS. In the sera from patients afflicted with Rheumatoid arthritis (RA), another autoimmune disease, antibodies have been found that reacted with the citrulline containing protein filaggrin. These antibodies reacted specifically to synthetic peptides containing citrulline but not to the arginine containing peptides (Schellekens et al., 1998). The investigators suggest that presence of these antibodies early in disease, before other signs occurred is indicative of a positive role for citrulline containing epitopes in inducing a secondary immune response in the pathogenesis of RA. In a study of proteins and epitopes recognized by antifilaggrin autoantibodies (AFA), these RA specific markers were shown to recognize deiminated proteins (Girbal-Neuhauser et al., 1999). Using filaggrin derived synthetic peptides, these authors also found that the presence of citrulline residues were constitutive of the AFA epitopes. Thus the presence of citrulline residues within filaggrin is an essential target for antibodies in the autoimmune response in RA. Since citrulline containing proteins can induce an immune response in RA and myelin from MS patients contains elevated levels of citrullinated MBP
(MBP-C8), these findings suggest that the causative agent for demyelination in MS may be the presence of high levels of MBP-C8, which is characteristic of a developmentally immature myelin. Therefore, the autoimmune response in MS appears to occur as a secondary stage of the disease targeted towards MBP-C8.

**E) Animal Models of Demyelinating Disease**

MS is a disease that involves multiple factors that function over a long period of time. This makes the study of the pathogenesis of MS difficult since the earliest indications of disease onset probably ensue well after the primary defect has occurred. Testing of treatment for MS in patients is complicated by the relapsing-remitting progression of the disease while treatments targeting the primary defect are virtually impossible. With this in mind, animal models have been created and utilized to study the pathogenesis of MS and to test drug treatments. The models used to date are viral models, autoimmune models (EAE), chemical models and a genetic model. Since remyelination occurs with the removal of the inductive agent in viral models, EAE, and chemical models and since demyelination in the genetic model develops and persists as the animals mature, the genetic model best resembles the persisting defect in MS.

1) Viral Models of MS

Due to their ability in rare cases to induce demyelination in the CNS, several viruses have been used to create a demyelinating system in animals to model MS. Viruses such as Theiler’s virus (Theiler’s murine encephalomyelitis virus, TMEV) (DalCanto and Lipton, 1979), mouse hepatitis virus (MHV), and Semliki forest virus (SFV) to name a few are commonly used to induce disease (Russell, 1997)(review). These models are effective in mimicking the induction of CNS inflammation, and the autoimmune response to disease. However, the effectiveness of infection is limited by the viral strain and the strain of animal
used. Some viruses, for example, TMEV and MHV are not directly neuroinvasive and neurotropic and thus require direct intracranial inoculation. The mechanism of infection also varies depending on the virus. Some infect oligodendrocytes and neurons while other viruses only infect other cells such as astrocytes. Although these models can duplicate some of the general characteristic of demyelinating disease, the variability in its effectiveness and in the mechanism leading to disease makes it difficult to discern the pathology for treatment of disease in MS.

II) Experimental Autoimmune Encephalomyelitis (EAE)

EAE is characterized by perivascular inflammation, infiltration of monocytes and lesions of demyelination. Disease is caused by an autoimmune response to the CNS initiated by immunization with autoantigens (active induction) or the transfer of T cells sensitized to myelin components (passive induction). First discovered by Rivers and Schwentker (Rivers and Schwentker, 1935) using monkeys, EAE is now more commonly induced in mice. Similar to the viral model of MS, different animal species and strains, chemical nature of antigen used, and the method of induction determines the severity of disease.

Immunization of animals can produce either acute EAE or chronic relapsing EAE (CREAE). Acute EAE is characterized by progressive paralysis of the hindlimb beginning within 10-14 days after injection of autoantigens such as MBP or MBP peptides in Freund's adjuvant. Infiltration of macrophages and CD4+ T cells is usually observed; however, demyelination does not often occur. If death does not ensue, these animals recover with no incidence of relapse. This type of EAE is particularity useful in testing therapeutic drugs. Disease in CREAE involves periodic attacks of acute EAE followed by phases of remission. Subsequent attacks increase in number and severity. CREAE is characterized by a mononuclear infiltrate, and primary demyelination. It has also been
found that epitope spreading can occur in CREAE in which autoreactivity induced by a primary antigen spreads to other proteins and peptides with subsequent relapses (Miller et al., 1995).

EAE antigens are injected with Freund's adjuvant (killed mycobacteria in a water-in-oil emulsion) to induce immunoactivity. They include: homogenate of whole brain or spinal cord, purified myelin, purified components of myelin, or peptides from myelin proteins. MBP in particular is a major encephalitogen (Kies and Alvord, 1959; Laatsch et al., 1962). The encephalitogenic epitopes in MBP occur at residues 1-11 (Zamvil et al., 1986) and 89-101 (Sakai et al., 1988). PLP and MOG have also been found to induce EAE (Hashim et al., 1980a; Hashim et al., 1980b; Madrid et al., 1982; Lington et al., 1992). The major epitopes in PLP occur in residues 139-151 (Tuohy et al., 1989) and 178-191 (Greer et al., 1992). Some non-myelin antigens can also induce EAE, for example, s100β from astrocytes (Kojima et al., 1994) and the small heat shock protein αB-crystallin from oligodendrocytes (VanNoort et al., 1995). These antigens all result in the activation of T cells which results in the autoimmune response in EAE. Since these antigens are found in the CNS, how these antigens penetrate the blood brain barrier (BBB) to activate T cells remains to be answered? Although EAE is commonly used as a model to study the disease process in MS, it only represents the immunoreactive aspect in demyelinating disease.

III) Chemical Models

Demyelination can also be induced in animals by injection or oral administration of ethidium bromide or cuprizone (Carlton, 1969; Blakemore, 1973; Ludwin, 1978). These models are characterized by degeneration of oligodendrocytes followed by demyelination and phagocytosis of myelin fragments. Axonal damage is also often observed. Since large
localized lesions that tend to recover occur without persisting defects, this model is particularly useful for the study of remyelination.

IV) Genetic Model

Both the viral and EAE models are based on an immunoreactive response. However, these model do not address the biochemical changes found to occur in MS. Since the myelin from patients suffering from MS is developmentally immature, a model that mimics this early myelinating system is required. As discussed earlier, DM20 protein is thought to play a role in cell differentiation and high levels of DM20 protein is a characteristic of developmentally immature myelin. By creating an animal that over expresses DM20, we would be able to model an early myelinating system. Using transgenic technology to insert a human DM20 cDNA transgene into a normal mouse background, several transgenic mouse lines containing from 2 to 70 copies of the transgene were produced (Simons-Johnson et al., 1995). The transgene is composed of a human cDNA under the transcriptional control of a normal human PLP promoter (Fig. 1.14). Attached at the 3’-end is an SV40 sequence containing a polyadenylation site for the stability of the mRNA. The major transcript from this transgene is 1.7kb while a minor component at 1.25kb can also be detected. Of interest is the transgenic mouse line containing 70 copies of this transgene (ND4). Mice from this line have been characterized and found to be spontaneously demyelinating (Mastronardi et al., 1993).

Developmental Profile of ND4 Mice

The ND4 mice behaved and developed normally from birth to 3 months of age. After 3 months of age, signs of demyelinating disease such as tremors, seizures, and wobbly gait were evident. These signs became progressively worse until 8-10 months of age when death ensued. However, these mice were able to produce offspring that carried
Figure 1.14: DM20 transgene construct and transgenic mouse lines. A) DM20 transgene consists of the following: a 2.95 kb normal human PLP gene promoter sequence; a 0.93 kb human DM20 cDNA; a 0.87 kb sequence from an SV40 vector containing a polyadenylation site (long arrow) and an intron (short arrow) (Simons-Johnson et al, 1995). This transgene only produces DM20 coding RNA. B) Northern blot of RNA isolated from normal and ND4 transgenic mouse brain probed for PLP+DM20 mRNA. The PLP gene transcripts resolve at 3.2 and 2.4 kb while the transgene transcripts resolve at 1.75 and 1.25 kb. C) Transgenic mouse lines produced with varying copy number of DM20 transgene.
the same genotype and developmental phenotype as their parents. EM studies of the optic nerves and white matter tracts from transgenic mice demonstrated that myelination was normal before the onset of disease (up to 3 months of age) but by 8-10 months of age loosely myelinated and nude axons were observed (Mastronardi et al., 1993). The amount of myelin obtained from 10 month old transgenic mice was decreased to ~17% of normal levels. Astrogliosis, a hallmark of demyelinating disease was observed by increases in staining for glial fibrillary acidic protein (GFAP, a marker for astrocytes) in white matter tracts, in the number of stained cells in sections from both 3 and 10 month old transgenic mice, and in the amount of GFAP quantitated in brain homogenate from 2 month old ND4 mice. Astrocytes containing myelin debris were also found in EM sections and lymphocytic infiltration was routinely seen in sections from ND4 mouse brain.

PLP and DM20 Protein in ND4 Mice

When the levels of PLP and DM20 proteins were measured, the developmental expression patterns for both proteins were reversed compared with normal mice. In the myelin from ND4 mice, the amount of PLP protein at 1 month of age was comparable to the levels found in 1 month old normal mice. Instead of increasing, the amount of PLP in myelin decreased with age and leveled off at approximately one tenth of normal levels (Barrese et al., 1998). The same decrease was observed in whole brain homogenate (Mastronardi, 1996). At one month of age, DM20 protein was virtually undetectable in myelin from transgenic mice (Barrese et al., 1998). Since DM20 was found to be in abundance at this age in whole brain homogenate from ND4 mice, this result suggested that the DM20 protein may not be assembled into myelin in ND4 mice at this age. After 1 month of age, the amount of DM20 in ND4 mice increased to approximately 3 times the amount found in normal mice (Mastronardi, 1996; Barrese et al., 1998). The myelin of
adult ND4 mice was characterized by high levels of DM20 and low levels of PLP which resembled a developmentally immature type myelin.

**MBP in ND4 Mice**

A developmentally immature myelin was also characterized by the profile of MBP in ND4 mice (Mastronardi et al., 1996b). The total amount of MBP was not decreased but the microheterogeneity was affected. The citrullinated form of MBP (C8) was first detected in brain cryosections using anti-citrulline antibody and found to be increased in 10 month old ND4 mice. This increase was also demonstrated by measuring the C8/C1 ratio in MBP from 10 month old normal and ND4 mice fractionated by alkaline-urea tube gels. The C8/C1 ratio was increased ~9 fold in ND4 mice. PAD activity was also measured in whole brain homogenate and found to be increased in ND4 mice (Mastronardi et al., 1996b).

Myelin in patients suffering from MS was characterized to be developmentally immature by a lower phase transition temperature and high levels of MBP-C8 relative to MBP-C1. The myelin from ND4 transgenic mice was also found to be developmentally immature. This was characterized by high levels of MBP-C8 and by increased amounts of DM20 and reduced levels of PLP. Since the ND4 mice are spontaneously demyelinating animals and since the myelin in these mice resembles the myelin from MS patients, the ND4 transgenic mouse is a good model in which to study the pathogenesis of disease and the effectiveness of therapeutic drug on demyelination.

**F) Therapeutic Treatments for MS**

Therapies for MS can be divided into three general categories: 1) treatment to prevent continuing destruction of tissue in the CNS by inflammatory cells; 2) treatment of symptoms to allow for the damaged nervous system to function at a higher level; 3) treatment to repair damaged CNS myelin. The majority of therapies for MS have been
directed towards the autoimmune aspect of the disease. Depending on the stage of disease, different drugs and regimens are employed. Corticosteroids are often used to treat acute cases of MS. These drugs target disabling symptoms but do not affect mild sensory attacks and have no effect on pathogenesis. In a recent study using high doses of the corticosteroid, methylprednisolone, the rate of development of MS was reduced over a three year span (Kupersmith et al., 1994). However, side effects such as mental changes, gastric disturbances, infections, increased risk to fractures, anaphylactic reactions, and arrhythmias were often seen with high dose usage.

A recombinant form of interferon-β-1b (betaseron) is currently used in the treatment of relapsing-remitting MS. Although this drug does not eliminate the repeated attacks, it does reduce the number of lesions detected by magnetic resonance imaging (MRI) (Paty et al., 1993) and the rate of disease progression (Sibley et al., 1995). A glycosylated analog of betaseron has been found to be more effective with less side effects. Other treatments of relapsing-remitting MS such as Azathioprine and Copolymer-1 (Copaxone) are not as effective and have greater side effects that limit their widespread use.

Due to the variability in response to treatment, treatment of chronic-progressive MS has been proven to be the most difficult. The common regimen is a pre-treatment stage with corticosteroids followed by pulse treatments with cyclophosphamide with corticosteroids. Other drugs such as cyclosporin and methotrexate have been tested but they are limited by their risk to benefit ratio (Rudge et al., 1989; Group-TMSS, 1990; Goodkin et al., 1995). A more vigorous approach for the treatment of chronic-progressive MS is hindered by toxicity.

The limitations of high toxicity on effective treatment affects all the drugs used in the treatment of MS. The major challenge of therapy is to have a drug that can be given early in disease to target primary lesions but has minimal toxicity with long term use.
G) Paclitaxel as a Drug Treatment for MS

The pathogenesis of disease in MS is complex and may involve several different processes. Some of these processes have been presented in this thesis which may serve as targets for drug treatment. The effectiveness of a therapeutic drug may be increased by targeting several, if not all, of these processes at the same time while maintaining minimal toxicity. Paclitaxel (Taxol), a well tolerated drug used in the treatment of ovarian and breast cancer (Rowinsky and Donehower, 1991), is an attractive candidate as a treatment for MS because of its potential to target multiple sites of action in the mechanism of demyelination. Originally isolated from the Pacific Yew tree (Taxus brevifolia) (Fig. 1.15) (Wani et al., 1971), paclitaxel is best known for its antimitotic effect on proliferating cells.

![Structure of paclitaxel](image)

**Figure 1.15**: Structure of paclitaxel.

This is facilitated by the binding of paclitaxel to the N-terminus of β-tubulin (Horwitz, 1992; Rao et al., 1994) which promotes assembly and prevents disassembly of microtubule polymers (Schiff et al., 1979). In this manner, paclitaxel can inhibit proliferating astrocytes which act as macrophages in the process of demyelination. Removal of proliferating astrocytes may promote recovery since these cells have been implicated in preventing remyelination in MS tissue by engulfing oligodendrocyte precursor cells (Prineas et al., 1990) and by wrapping processes around denuded axons (Ludwin,
1995). Paclitaxel has also been shown to inhibit the expression of matrix metalloproteinases (MMPs) in cultured chondrocytes (Hui et al., 1998). This family of endoproteases has been implicated in MS by the increase in their expression and activity in tissue and CSF from MS patients (Gijbels et al., 1992; Gijbels et al., 1993; Gijbels and Steinman, 1994a; Maeda and Sobel, 1996) and also by their activity on MBP as a substrate (Gijbels et al., 1993; Chandler et al., 1995). Alternatively, paclitaxel was found to inhibit PAD activity on benzoylarginine ethyl ester (BAEE) and MBP in vitro (Pritzker and Moscarello, 1998). The inhibitory effect of paclitaxel on the activity of PAD can be argued to arise from non-specific interactions between the drug and the enzyme. It is believed that at high concentrations of paclitaxel, access to PAD is essentially blocked by excessive interaction of the drug with PAD. However, when PAD was incubated in excess tritiated paclitaxel (10^4 excess paclitaxel over PAD) and passed through a Sephadex G-100 gel filtration column, PAD was shown to elute much earlier than the labeled paclitaxel and to contain less than 0.1% of tritiated paclitaxel (Pritzker and Moscarello, 1998). This indicated that with high concentrations of paclitaxel, not all of the drug interacted with PAD and suggests that paclitaxel bound specifically to PAD. Therefore, the inhibitory effect of paclitaxel on PAD activity cannot be attributed to non-specific binding at high concentrations of the drug. As discussed in an earlier section, increased PAD may be responsible for the high levels of MBP-C8 found in myelin from MS patients. A decrease in its activity may reduce the levels of MBP-C8 which may reduce the extent of myelin destabilization. Lastly, Whitaker et al. found that paclitaxel can also inhibit the uptake of ^3H-thymidine by MBP activated lymphocytes (J. Whitaker, personal communication). This finding implies that paclitaxel can inhibit the proliferation of lymphocytes targeted to myelin. Through these actions and its tolerance and low risk side effects in cancer patients, paclitaxel may be an effective therapeutic agent for the treatment of demyelinating disease.
Hypothesis and Objectives

The myelin from patients suffering from MS and from the spontaneously demyelinating animal model, the ND4 transgenic mouse, has been shown to be developmentally immature. This is characterized by the change in biochemical profile of myelin proteins and the similarities in physical structure reported by Chia et. al. (Chia et al., 1984). Based on these findings, it is hypothesized that demyelination in the ND4 mouse arises from myelin that is maintained at an early myelinating stage in which it is less compact. Although the ND4 mice can tolerate this form of myelin initially, this less developed myelin and its components become increasingly susceptible to destabilization with age.

Using the ND4 mice as a model for demyelinating disease, the objectives of this thesis are: 1) to elucidate and to identify the biochemical mechanism responsible for myelin destabilization; 2) to test the effectiveness of paclitaxel as a therapeutic agent for demyelinating disease.
Chapter 2: Experimental Procedures

Materials

The enzymes used for the different molecular biology techniques were purchased from different companies: Proteinase K was from Boehringer Mannheim; RNase A, restriction enzyme Bgl II, T4 polynucleotide kinase were from Pharmacia Biotech. Reagents used in these techniques included phenol: water (90:1) (Gibco BRL), N-lauroylsarcosine (Sigma), Spermine (Sigma), Spermidine (Sigma), Poly dI-dC·dI-dC (Pharmacia Biotech). Guanidinium isothiocyanate (GuSCN) (Fluka), Cesium Chloride (CsCl) (Kodak), Diethylpyrocarbonate (DEPC) (ICN), Denhardt’s solution (Sigma), and Salmon sperm DNA (Pharmacia Biotech). Membranes used for Southern blot, Northern blot and Western blot were nitrocellulose (Pharmacia Biotech) and Hybond-N+ (Amersham). Radionucleotides used for the labeling of probes were γ-32P ATP from ICN and α-32P dCTP from Mandel. The Ready-to-go labelling kit was purchased from Pharmacia Biotech which was used to label the cDNA probes including the PLP cDNA (pMD14), cyclophilin cDNA (gift from Dr. A. Roach, (Milner et al., 1985)), c-Fos cDNA, c-Jun cDNA, stromelysin-1 cDNA and the GAPDH cDNA (gifts from Dr. T. Cruz, Dept. of Pathology, Mount Sinai hospital, Toronto), and TIMP-1 cDNA (gift from Dr. D. Edwards, Calgary, Alberta). Oligo-1 and Oligo-4 probes used for gel mobility shift assays and the ΔPLP probe used for the Northern blots were synthesized at the Biotechnology Service Center (Hospital for Sick Children, Toronto).

Hank’s solution, essential amino acid mix, non-essential amino acid mix, 1X vitamin mix, penicillin/streptavidin antibiotics and lipoic acid used in the RNA stability assay experiments were purchased from ICN. Other reagents such as L-glutamine and
ascorbic acid were from Sigma while 5,6-dichlorobenzimidazole-1β-D ribofuranoside (DRB), a transcription inhibitor, was from Fluka.

CM-Tris acryl resin used for the separation of DM20 and PLP was purchased from Biospra. Primary antibodies used for Western blots were C-terminal PLP (CT-PLP/Ab105 (Fannon et al., 1994) and ΔDM20 (Serotec) to detect PLP and anti-GFAP antibody (DAKO) to detect glial fibrillary acidic protein (GFAP). Secondary antibodies used included goat-anti-rabbit IgG and goat-anti-mouse IgG (Biorad). Reagents used in the colorimetric reaction included 4-chloro-1-naphthol (4-CN) (ICN) and N, N-diethyl-p-phenylene-diamine (DEPDA) (Sigma). GFAP from bovine spinal cord used for the slot blot standard curve was from American Research Products, Inc..

Pentadecanoic acid, palmitate, stearate and pentadecanoate used in the GC/MS experiment were purchased from Sigma. N,O,-Bis-(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane were bought from Pierce.

Equipment used included the slot-blot apparatus (Biorad), Phosphor screen and image scanner (Molecular Dynamics), GC/MS (HP-5 fused silica column (Hewlett Packard) and VG Trio mass spectrophotometer (VG Mass Lab-Fusion Instruments)), Oligo 1000M DNA synthesizer (Beckman Instruments Inc), and a Hitachi H500 electron microscope (Hitachi).

Normal and ND4 transgenic mice used were of CD1 background. These were housed in the animal facilities unit in the Banting and Best institute at the university of Toronto (Canada). Transgenic mice were prepared by Simons-Johnson et. al. (Simons-Johnson et al., 1995) of our institution. Briefly, the cDNA for human DM20 was incorporated into the genome of a normal mouse CD-1 background. Several lines were produced with different copy numbers of the DM20 transgene. These included the ND2
and ND5 line which had 4 copies of the transgene, the ND3b line with 2 copies, the ND3a line with 17 copies and the ND4 line with 70 copies. Only mice from the ND4 line were used in the studies presented in this thesis. Transgenic mice were screened by Southern blot analysis on DNA extracted from mouse tail. The different lines were maintained by breeding transgenic mice with normal mice whose transgenic off-spring displayed the same genotype and phenotype as their transgenic parent.

TIMP-1 overexpressing transgenic mice were gifts from Dr. R. Khokha (Dept. of Biophysics, University of Toronto, Toronto). These animals were house in the same facilities as the ND4 transgenic mice.
Methods

A. Molecular Biology Procedures

i) Analysis of ND4 Transgenic Mice

Isolation of Genomic DNA from Mouse Tails

To determine which newborn mice carried the DM20 transgene, Southern blot analysis was done on the DNA isolated from tail clips. Tails clipped from mice were diced using scissors in 700 μl of proteinase K buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% SDS). Proteinase K (Boehringer Mannheim) (35 μl of a 10 mg/ml stock) was added and the suspension was incubated overnight at 55-60° C. The next morning, the mixture was treated with 20 μl of RNase A (Pharmacia Biotech) (10 mg/ml) for 2 hrs at 37° C and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) followed by one extraction with chloroform/isoamyl alcohol (24:1, v/v). The DNA was precipitated by addition of 100% isopropanol and spooled onto a glass rod. The glass rod containing DNA was dehydrated by washing with 70% ethanol once followed by two washes with 95% ethanol. The DNA was then dried under vacuum and redissolved overnight in 400 μl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). DNA concentrations were determined by measuring the absorbance at 260 nm.

Southern Blot Analysis of DNA from Mouse Tails

Approximately 5 μg of genomic DNA isolated from mouse tails was digested overnight with 2 μl Bgl II (50 U/μl) (Pharmacia Biotech) in a 37° C water bath. The next morning, 1 μl of Bgl II was added and the digestion was allowed to continue for another 2 hrs after which time the DNA fragments were analyzed by Southern blot as follows.

The Bgl II digested genomic DNA was separated by electrophoresis through an 8% agarose gel containing TBE pH 7.5 (89 mM Tris, 89 mM boric acid, 0.02 M EDTA) at 80V for 2-3 hours or until the dye front reached 3/4 down the length of the gel. The
DNA was prepared for transfer by soaking the gel in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 hr with four changes followed by neutralizing buffer (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) for 1 hr with four changes. DNA was transferred onto nitrocellulose membrane overnight. The following day, the filter was baked in an 80°C oven for 1 hr, prehybridized with hybridization buffer (5X SSC (0.75 M NaCl, 0.75 M Na-citrate), 0.1% N-lauroylsarcosine (Na Salt), 0.02% SDS, 1% Blotto) for 1 hr in a 65°C water bath and then hybridized overnight with hybridization buffer containing 32P-labeled PLP cDNA probe (pMD14, gift from Dr. R. Roach (Milner et al., 1985)) in a 65°C shaking water bath. The next day, the filter was washed twice at 65°C with wash I (2X SSC, 0.1% SDS), twice with wash II (0.1X SSC, 0.1% SDS), air dried and exposed to film.

Mouse genomic DNA was also analyzed by Southern slot blot. Undigested genomic DNA was mixed with 100 µl of denaturing solution (0.4 M NaOH, 10 mM EDTA), boiled for 10 min and neutralized with 100 µl of cold neutralizing solution (2 M Ammonium acetate pH 7.0). Nitrocellulose membrane was pre-soaked in 6X SSC, placed in the slot blot apparatus (Biorad) and washed under vacuum with 500 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). The DNA samples were loaded into individual slots and applied to the membrane under vacuum. The membrane was then washed with 2X SSC, air dried and baked for hybridization as described above.

**ii) Transcription Factor Assay**

**Preparation of Brain Nuclear Extract**

Brain nuclear extract from normal and transgenic mice was prepared as described in Berndt et al. (Berndt et al., 1992). Briefly, nuclei from whole mouse brains were prepared first by homogenizing 1g of tissue in 5 ml of homogenization buffer (10 mM Hepes buffer pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol). The homogenate was divided into two parts and each part
was layered over 1.25 ml of homogenization buffer in a 5 ml ultracentrifugation tube. Each gradient was centrifuged for 30 min at 28,000 rpm, 4°C in an SW55Ti rotor to pellet the nuclei.

To prepare the extract, nuclei were lysed as follows. The pelleted nuclei were dislodged from the centrifuge tube with a spatula, suspended in 500 μl of lysis buffer (20 mM Tris-HCl pH 7.9, 0.42 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) and lysed using a Dounce homogenizer. Low salt nuclear extract was prepared by the same method except the lysis buffer consisted of 20 mM Tris-HCl pH 7.9, 0.1 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol. The lysate was transferred to an Eppendorf tube and mixed for 30 min at 4°C. After this, the mixture was centrifuged for 10 min to precipitate the DNA. The supernatant was removed and dialyzed against 50 volumes of buffer (20 mM Tris-HCl pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) for 16 hrs at 4°C. Protein content was determined using the Bradford assay (Bradford, 1976).

*Gel Mobility Shift Assay (GMSA)*

For GMSA reactions, 1-4 μg of nuclear extract were incubated with 2 μl of ³²P-end-labeled, double-stranded oligonucleotide (Oligo 1 and Oligo 4, (Berndt et al., 1992) and 4 μl Poly (dI-dC)-(dI-dC) in a final volume of 20 μl of buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 100 mM β-mercaptoethanol, 0.1% Triton X-100 and 4% glycerol). For competitive GMSA reactions, the mixture was incubated with 10-fold, 50-fold or 100-fold excess of unlabeled oligonucleotide for 15 minutes. DNA-protein complexes were resolved on a pre-electrophoresed (2 1/2 hrs at 100 V) 6% polyacrylamide non-denaturing gel (25 mM Tris-HCl pH 8.5, 190 mM Glycine, 1 mM EDTA) run at 125V for 2 hrs. The gel was dried and exposed to a Phosphor screen (Molecular Dynamics) to quantitate the band intensities.
iii) Isolation and Analysis of RNA in Mouse Brain

Isolation of RNA from Mouse Brain

RNA was extracted from mouse brain from normal and ND4 transgenic mice ranging in age from two weeks to 8 months by the method of Chirgwin et al. (Chirgwin et al., 1979). One whole mouse brain was homogenized in 5 ml of guanidinium isothiocyanate (GuSCN) solution (4.2 M GuSCN, 60 mM Na-Acetate pH 6.5, 0.1% sarkosyl, 25 mM EDTA pH 8.0, 100 mM 2-mercaptoethanol). The homogenate was layered over a 2 ml cesium chloride cushion (5.7 M CsCl, 50 μM Na-Acetate pH 6.5, 1 mM EDTA) and centrifuged at 32,000 rpm, 15° C, for 18-20 hrs in an SW41 rotor. Once the centrifugation was completed, the supernatant was carefully removed without disturbing the RNA pellet. The pellet was resolubilized with two 20 min washes with diethylpyrocarbonate (DEPC)-treated H2O (200 μl each) on ice. These washes were pooled, transferred into an Eppendorf tube, and the RNA was precipitated with the addition of 40 μl of 3 M Na-Acetate (pH 6.5) and 800 μl of ethanol. The RNA was recovered as a pellet by centrifugation for ~15 min. This pellet was washed with 70% cold ethanol, dried briefly to remove residual ethanol, and redissolved in 200 μl of DEPC-H2O. By measuring the absorbance at 260 nm, the amount of RNA was calculated from the following formula: 1 OD at 260 nm = 40 μg/ml of RNA (Maniatis et al., 1982).

Northern Blot Analysis

Whole brain RNA was resolved on a 1.2% agarose formaldehyde gel and transferred to Hybond-N+ membrane (Amersham) with 3.0 M NaCl, 0.3 M Na-citrate (20x SSC). The membrane was blocked with prehybridization solution (10% dextran sulfate, 5x sodium chloride, sodium phosphate, 5 X SSPE (0.9 M NaCl, 0.05 M Na phosphate buffer pH 7.7, 5 mM EDTA), 50% formamide, 5.73X Denhardt's solution, 4.2
mg salmon DNA (0.28mg/ml) and 0.5% SDS) and hybridized with either 32P-labeled cDNA or oligonucleotide probe. Following this, the membrane was washed twice with wash 1 (2x SSC, 0.1% SDS) at 65°C and then with wash 2 (0.1x SSC, 0.1% SDS) at room temperature. The filter was dried and was exposed to a Phosphor screen (Molecular Dynamics). Quantitative data from the Northern blots were normalized to cyclophillin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA.

For the developmental expression of PLP and DM20 mRNA, the probes used were a synthetic oligonucleotide specific to exon 3b in the PLP mRNA (ΔPLP) and a PLP and DM20 specific probe (rat PLP cDNA, pMD14). The rat PLP cDNA probe was isolated from the pMD14 plasmid.

iv) RNA Stability Assay

Stability of RNA was measured by the method of Johnson and Keeley (1990) (Johnson and Keeley, 1990). The brain from normal and transgenic mice was dissected and rinsed quickly with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2). Each brain was sectioned into quarters with a sterile scalpel and placed in a 25 ml culture flask with 10 ml of filter membrane sterilized media containing 60 μM 5,6-dichlorobenzimidazole-1β-D ribofuranoside (DRB), a transcription inhibitor, (Fluka). The media was composed of 1X Hank’s salt solution, 1X essential amino acid mix, 3X nonessential amino acid mix, 2 mM L-glutamine, 0.28 mM ascorbic acid, 40 mM Hepes, 1X vitamin mix, 2% penicillin/streptavidin antibiotics, and lipoic acid pH 7.6.

Each culture was incubated in a 37°C shaking water bath. The flasks were placed just above the water level so that the cultures were not contaminated by the water. The caps to each flask were loosely threaded to allow air to pass into the flask and to facilitate passive diffusion of oxygen into the tissue. At various time points, the tissue was
removed from the media for RNA extraction and Northern blotting. The Northern blot was probed with a $^{32}$P-labeled PLP cDNA (pMD14, (Milner et al., 1985)) and the band intensities were obtained using an Image Quant phosphor screen.

v) **Radioactive Labelling of Probes**

*5'-End Labelling of DNA Probes*

Synthetic oligonucleotide probes used for GMSA and Northern blots were synthesized at the Biotechnology Service Center (Hospital for Sick Children, Toronto) on an Oligo 1000M DNA synthesizer (Beckman Instruments Inc) and cartridge dried. These probes were 5'-end labelled with $\alpha$-$^{32}$P-ATP (ICN) as follows. Approximately 1 µg of oligonucleotide was mixed with 2 µl of 5X buffer mix (0.25 M Tris pH 7.6, 50 mM MgCl$_2$, 25 mM DTT, 0.5 mM spermidine HCl, 0.5 mM EDTA), 5 µl of $\gamma$-$^{32}$P-ATP (25 µCi), 1 µl of T4 Polynucleotide Kinase, and H$_2$O in a volume of 10 µl and then incubated at 37° C for 30 min. After the incubation, the mixture was heated at 65-70° C and allowed to cool slowly.

For the GMSA probes, the labelled DNA was resolved on a 20% acrylamide gel containing TBE pH 8.0 (89 mM Tris-Borate, 89 mM boric acid, 0.02 M EDTA) at 125 V for 1hr. DNA was visualized by staining the gel in TBE containing ethidium bromide (0.5 µg/ml) for 15 min and excised from the gel, diced and eluted into 200-400 µl of dH$_2$O overnight. Extent of labelling was measured on a $\beta$-counter.

For the Northern blot probe (APLP), the reaction mixture was fractionated on a G-50 Sephadex column equilibrated with TES buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS). Aliquots from each fraction were measured on a $\beta$-counter (Beckman) and the fractions containing labelled probe (the first peak) were pooled.
Random Primer Labelling of DNA Probes

Random primer labelling was performed using the Ready-To-Go DNA labelling kit (-dCTP) from Pharmacia Biotech. Briefly, the labelling mix (dATP, dGTP, dTTP, FPLCpure Klenow fragment, random oligodeoxyribonucleotides (9-mers) dried in buffer) was resolubilized with 25 \mu l of ddH2O on ice for 30 min. Meanwhile, 1-25 ng of cDNA was adjusted to a volume of 20 \mu l with ddH2O, boiled for at least 3 min and then quickly placed on ice. The cooled cDNA and 5 \mu l of \alpha-32P-dCTP (100 \mu Ci/\mu l) (Mandel) was added to the resolubilized labelling mix and the reaction mix was incubated in a 37\(^\circ\) C water bath for at least 1 hr. The labelled probe was separated from the reactants by gel filtration on a G-50 Sephadex column equilibrated with TES buffer. Aliquots from each fraction were measured on a \beta-counter (Beckman) and the fractions containing labelled probe (the first peak) were pooled.

B) Analysis of Covalently Bound Fatty Acids on PLP and DM20

i) Preparation of PLP and DM20 from Mouse Brain

Total PLP was prepared from whole mouse brain by homogenizing 20 brains in chloroform:methanol:HCl (2:1:5\% of 0.1N, v/v/v) in a proportion of 0.12g/ml. This was mixed overnight at 4\(^\circ\) C and was filtered through 1mm Whatman paper the following day. Three volumes of ether was added to the filtrate. This was left at -20\(^\circ\) C overnight to precipitate total proteolipids. The proteolipids were recovered as a pellet by centrifugation in a JA 20 rotor at 8000 rpm, -10\(^\circ\) C for 20 min and was redissolved in chloroform:methanol:water (4:4:1, v/v/v) for fractionation on CM-Tris acryl column.
**ii) Fractionation of PLP and DM20 on CM- Tris acryl Column**

PLP and DM20 was fractionated on a CM- acryl column (BioSepra) (Helnyck et al., 1983). A slurry of the CM-Tris acryl resin was prepared in chloroform:methanol:water (4:4:1, v/v/v) and poured into an acid washed column (0.4 x 10 cm). The matrix was allowed to pack under gravity and the column was washed with chloroform:methanol:water (4:4:1, v/v/v). On each column, ~10 OD$_{280nm}$ units of total PLP were applied. The column was washed with 18 ml of chloroform:methanol:water (4:4:1, v/v/v) and eluted with 36 ml of 0.01 M ammonium acetate in chloroform:methanol:water (4:4:1, v/v/v) followed by 50 ml of 0.05 M ammonium acetate in chloroform:methanol:water (4:4:1, v/v/v). Each fraction was collected in 2 ml portions and the absorbance at 280 nm was measured for each fraction.

DM20 protein eluted with 0.01 M ammonium acetate in chloroform:methanol:water (4:4:1, v/v/v) in fractions 15 to 17 and was identified by Western Blot using antibody to residues 271-276 (C-terminal PLP) and amino acid analysis. PLP eluted with 0.05 M ammonium acetate in chloroform:methanol:water (4:4:1, v/v/v) in fractions 40 to 43. This was further purified by precipitation with 5 volumes of cold acetone and separation on a preparative SDS gel. The gel was presoaked in 2.5% triton X-100 to remove SDS and the band corresponding to the Mr corresponding to PLP was excised, minced and homogenized in chloroform:methanol:HCl (2:1:5% of 0.1N, v/v/v). After overnight elution, the supernatant was recovered by centrifugation and PLP in the supernatant was identified by Western blot using antibody specific to residues 116-150 (ΔDM20) and by amino acid analysis.
Western Blot

Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred onto nitrocellulose membranes for detection by the method of Towbin et al. (1979) (Towbin et al., 1979). Briefly, after the SDS-PAGE was completed, the proteins were transferred onto nitrocellulose membranes electrophoretically in cold 1X transfer buffer (10% 10X transfer buffer (250 mM-HCl pH 8.0, 1.92 M glycine, 0.3% SDS) and 20% methanol in water) for 1 hr at 300 mA. The membrane was rinsed with TBS, blocked for 1 hr with blocking solution (5% carnation milk powder dissolved in TBS (w/v)) and incubated overnight at 4°C with the primary antibody (polyclonal antibody to residues 200-224 of PLP (CT-PLP/Ab 105) or antibody to residues 116-150 in PLP (ΔDM20)). The following day, the blot was washed twice with TTBS (0.05% Tween-20 in TBS (v/v)) (5 min each), twice with TBS (5 min each), and then incubated for 1 hr at room temperature with either goat anti-rabbit IgG conjugated to horse-radish peroxidase (HRP) (for polyclonal antibody) or goat anti-mouse IgG conjugated to HRP (for monoclonal antibody). The blot was washed again with TTBS and TBS as described and antibody binding was detected by adding the colorimetric detection reagent followed by 2 μl of H2O2. The colorimetric detection reagent was made by adding 250 μl of colour developer (10 mg of 4-chloro-1-naphthol and 6.25 mg N, N-diethyl-p-phenylene-diamine (DEPDA) dissolved into 500 μl of methanol) to 12.5 ml of 0.1 M Citrate buffer pH 6.0 (88.4 mM Na citrate, 12.4 mM citric acid). The colorimetric reaction was stopped by replacing the solution with water.

iii) Quantitation of Palmitate and Stearate in DM20 and PLP by Tandem Gas Chromatography/ Mass Spectrometry (GC/MS)

For fatty acid analysis, both PLP and DM20 were dried under nitrogen and hydrolyzed in 6 N HCl at 110°C for 24 hrs in gas phase. To each hydrolysate, 50 μl of
10 mM pentadecanoic acid in methanol was added as an internal standard, followed by an additional 1 ml of methanol and vortexed. To extract the fatty acids, each solution was centrifuged at 13,000 rpm for 5 min in an Eppendorf Microcentrifuge and the methanol was transferred to another tube. After two more extractions with 1 ml of methanol, the three methanolic extracts were combined and evaporated to dryness under nitrogen. Trisethylsilyl derivatives were formed by adding 100 µl N,O-Bis-(trisethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane and carrying out the reaction at 60°C for 20 min. Derivatized samples used for the determination of stearate and palmitate content were diluted to 1:10 with n-hexane. Since N-methyl glycine precipitated in n-hexane, derivatized samples and standard containing N-methyl glycine used to measure N-methyl glycine were not diluted. A standard mixture containing palmitate (250 nmol), stearate (250 nmol) and pentadecanoate (500 nmol) were derivatized and extracted into n-hexane as described above.

Quantitation of samples was performed by capillary gas chromatography/mass spectrometry (GC/MS, Hewlett Packard) equipped with a HP-5 fused silica column (30m X 0.32mm) interfaced with a VG Trio-2A quadrapole mass spectrometer (VG Mass Lab-Fusion Instruments) equipped with Lab-Base™ data system. The column phase was cross-linked 5% phenylmethylsilicone gum, 1.0 mm film thickness. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. One microliter of the standard (known quantities of palmitate, stearate, N-methyl glycine and pentadecanoate) and of each sample was injected in the splitless mode under the following conditions: initial oven temperature 60°C, initial time 0.5 min, ramp rate 20°C/min, second oven temperature 200°C, ramp rate 8.00°C/min, final temperature 285°C, injector temperature 250°C. The standard mixture and each sample were analysed on separate runs on the same day. Quantitation of palmitate, stearate and N-methyl glycine was performed in positive electron impact ionization (EI+). Each species including the internal standard was
measured in the selected ion recording mode with a scanning dwell time of 80 msec. The (M-15)+ ions at m/z= 313 (C16:0), m/z=341 (C18:0), m/z=116 (N-methylglycine) and m/z=299 (pentadecanoic acid) were scanned.

C) Procedures used in the Analysis of Paclitaxel Treated ND4 Transgenic Mice

i) Paclitaxel Treatment of ND4 Transgenic mice

Paclitaxel was provided as a water-soluble preparation in micelles of methoxypolyethylene glycol (Angiotech, Vancouver) in a weight ratio of 10% paclitaxel in micelles. This was prepared by melting micellar preparation in a 55-60°C water bath and then solubilizing to a concentration of 16 mg/ml in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2).

Normal and ND4 transgenic mice were treated with solubilized paclitaxel at 3-3.5 months of age (with the onset of clinical signs) or at 2-2.5 months of age (before the onset of clinical signs). Paclitaxel was injected intraperitoneally with a dosage of 20 mg paclitaxel/kg body weight per mouse once every week for four weeks. Animals were observed and scored for the severity of shaking, head tremors, hindlimb tremors, jerky head, wobbly gait, and unsteadiness. These signs were scored on a four point system: 1+, mild signs; 2+, mild but consistent signs; 3+, severe signs; 4+, severe signs with loss of control. The clinical progression of each animal was scored three times a week beginning with the first injection until the mouse was sacrificed.
ii) **Light Microscopy of Brain Sections from Mice**

Sections were prepared as described by Ludwin (Ludwin, 1978). Epon-embedded section of 1μm in thickness were placed on slides and dipped in Luxol fast blue to stain for myelin. The samples were then examined by light microscopy.

iii) **Electron Microscopy of Brain and Optic Nerve Sections**

The brain and optic nerve from normal, ND4 transgenic and paclitaxel treated ND4 transgenic mice were freshly dissected and fixed in 5% glutaraldehyde in 0.05 M phosphate buffer pH 7.4 at 4°C. The tissue was post fixed in phosphate buffered osmium tetroxide for 1 hr. It was then washed in 0.05 M phosphate buffer, dehydrated in 25%, 50%, 70%, 90% and 100% ethanol and embedded in Epon-Araldite. Ultra thin sections were cut and mounted on copper grids which were then stained with 2% uranyl acetate and Reynold’s lead citrate. The stained sections were viewed and photographed with a JOEL 1200 ExII transmission electron microscope.

iv) **Analysis of Glial Fibrillary Acidic Protein (GFAP) in Mouse Brain**

*Electron Microscopic Autoradiography of Brain Sections from Mice*

Electron microscopic autoradiography was performed as described by Kopriwa et. al. (Kopriwa, 1973). Ultrathin sections were cut from Epon blocks and arranged in ribbons. These ribbons were placed onto slides coated with 0.8% celloidin solution (celloidin stock (Tissue embedding solution No. M4700, 30% in alcohol-ether (1:1), Randolph Products Company) diluted with isoamyl alcohol to 0.8%), dipped in and
coated with Ilford L4 emulsion and exposed in the dark for 10 weeks. The slides were developed in a Kodak D-19 developer solution (Kodak), washed with distilled water, fixed in 24% sodium thiosulfate and washed with five changes of distilled water, 1 minute each. The area containing the celloidin-section-emulsion complex was removed from the glass slide, transferred to EM grids, and dried. The grids were post-stained with uranyl acetate and lead citrate for examination with a Hitachi H500 electron microscope.

**Immunohistochemical Staining for GFAP in Brain Sections**

Brain, freshly dissected from normal, ND4 transgenic, and paclitaxel treated ND4 transgenic mice were fixed in 10% buffered formalin overnight and then embedded in paraffin. Sections of 5 μm thickness were cut, placed on silanated slides and dried in a 37°C - 50°C oven overnight. The following day, the sections were deparaffinized and hydrated with sequential immersion in 100%, 80%, 60% and 40% ethanol and then twice in ddH2O. Endogenous peroxidase was blocked by incubating the mounted sections with hydrogen peroxide (H2O2) in methanol (5 ml 30% H2O2 in 100 ml absolute methanol) for 30 min at room temperature. This solution was washed off with ddH2O and the sections were placed in normal goat serum (16 μg/ml) to block for 10 min. The sections were incubated for 1 hr at room temperature with polyclonal GFAP antibody (DAKO) diluted 1:1000 in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2) containing 5% bovine serum albumin (PBS-BSA) and washed three times with PBS-BSA (5 min each). They were then incubated with biotinylated goat anti-mouse IgG diluted 1:200 in PBS-BSA for 30 min at room temperature and washed three times with PBS-BSA (5 min each). Concurrently, the ABC elite stain (20 μl avidin, 20 μl biotin per 1 ml of PBS-BSA) was mixed and left
standing for 10 min before incubating it with the sections for 30 min at room temperature. Afterwards, the sections were washed with PBS-BSA, rinsed with 0.05 M-HCl pH 7.6 and stained again with fresh DAB solution (0.3% H₂O₂, 3,3'-diaminobenzidine at 0.3 mg/ml in 50 mM-HCl pH 7.5, 150 mM NaCl and 0.1% triton X-100) for 5 min at room temperature. The reaction was stopped by rinsing with H₂O and the sections were counter stained with haematoxylin for 1 min. The reaction was developed by rinsing in H₂O, dehydrating sequentially in 50%, 70%, 80% and 100% ethanol and dipping in 1% ammonia in H₂O for 10-20 sec. The samples were dehydrated in xylene and mounted with a coverslip.

Quantitation of GFAP in Mouse Whole Brain Homogenate

Whole brain from normal, ND4 transgenic and paclitaxel treated ND4 mice were homogenized in buffer containing 50 mM-HCl pH 7.6, 0.5 mM DTT, 1 mM EDTA, 0.43 mM PMSF and centrifuged to obtain the supernatant and the pellet. The pellet was resuspended in buffer containing 10 mM Na phosphate pH 7.5, 2 mM DTT, 6 M urea and 1 mM EDTA for GFAP quantitation. Protein concentrations were determined by Peterson assay (Peterson, 1977). Each sample was diluted to 0.125 ng/μl and 200 μl (25 ng of total protein) was loaded into each slot. On the same blot, a standard curve was prepared using purified GFAP from bovine spinal cord (American Research Products, Inc.) in concentrations from 0 to 100 ng. All samples were applied onto nitrocellulose membrane pre-wet in 1X TBS (1 mM-HCl pH 7.5, 1.5 M NaCl) using a slot blot apparatus (BioDot SF, Biorad) under vacuum.

After the proteins were transferred to nitrocellulose, the blot was blocked with blocking solution (5% carnation milk powder dissolved in TBS (w/v)) for 1 hr and
incubated overnight at 4° C with the primary antibody polyclonal anti-GFAP (DAKO). The following day, the blot was washed twice for 5 min each with TTBS (0.05% Tween-20 in TBS (v/v)), twice for 5 min each with TBS, and then incubated for 1 hr at room temperature with goat anti-rabbit IgG conjugated to horse-radish peroxidase. The blot was then washed twice for 5 min each with TTBS, twice for 5 min each with TBS and antibody binding was detected by adding the colorometric detection reagent followed by 2 μl of H₂O₂. The colorometric detection reagent was made by adding 250 μl of colour developer (10 mg of 4-chloro-1-naphthol and 6.25 mg N, N-diethyl-p-phenylene-diamine (DEPDA) dissolved into 500 μl of methanol) to 12.5 ml of 0.1 M Citrate buffer pH 6.0 (88.4 mM Na citrate, 12.4 mM citric acid). The absorbance from each band was obtained by scanning in a densitometer (LKB) at 633 nm. From the standard curve, the protein concentration was obtained and expressed as ng GFAP/ug of homogenate protein.

D) Other Analytical procedures

i) Amino Acid Analysis

Protein samples were dried under nitrogen and were hydrolyzed for 24 hours with 5.7 N HCl containing 0.1% phenol at 110° C in gas phase. The free amino acids were derivatized with phenylisothiocyanate (PITC) and analyzed on a Waters Pico-Tag™ automated amino acid analyzer (Heinrikson and Meredith, 1984).
ii) Phosphorus Assay

Phosphorus content in DPPC vesicles were measured as described by Bartlett (Bartlett, 1959). To an aliquot of DPPC vesicles dried in acid washed tubes, 0.3 ml of H$_2$SO$_4$ was added. Following this, three drops of 30% H$_2$O$_2$ was added. This mixture was heated for 15 mins and then left to cool. To this 7.5 ml water, 1.5 ml 2.5% ammonium molybdate and 0.2 ml Fiske-Subba Row reagent (25 mg 1-amino-2naphthol-4-sulfonic acid) was added with mixing in between each addition. Next, the mixture was boiled for 10 min, cooled and the absorbance was taken at 830 nm. The amount of phosphorus was determined from a standard curve made with phosphate.
Part I: Biochemical Reactions Leading to Myelin Instability in the ND4 Transgenic Mouse

The process of myelin breakdown in demyelinating diseases such as Multiple Sclerosis (MS) has long been considered to arise from an autoimmune response. However, recent findings have challenged this perception and placed the primary cause for demyelination at inherent defects occurring within myelin and myelin producing cells. Reviewed in the introduction, the most compelling results supporting this view have been reported in a series of experiments under the direction of Dr. M. A. Moscarello. Briefly, MBP isolated from myelin from MS patients was shown to contain higher levels of MBP-Cit6 relative to MBP-Cit0 characteristic of less mature myelin (Moscarello et al., 1994). In fulminating MS, the increase in MBP citrulline content corresponded with severity of disease (Wood et al., 1996). This change in MBP was not due to changes in autopsy material since MBP isolated from brain samples from other neurological diseases did not present the same modifications (Moscarello et al., 1994) nor was it the result of an autoimmune reaction since a normal profile for MBP charge isomers was found in EAE animals (Mastronardi et al., 1996a). However, the increase in the relative proportion of MBP-Cit6 was found in the ND4 transgenic mice (Mastronardi et al., 1996b), a spontaneously demyelinating animal (Mastronardi et al., 1993; Simons-Johnson et al., 1995). It was concluded that an alteration in the post-translational modification of MBP resulting in increased relative levels of MBP-Cit6 contributed to myelin breakdown.

In addition to changes in the levels of MBP charge isomer, the amount of PLP and DM20 protein were also altered in adult ND4 animals. Quantitation of PLP and DM20 protein in myelin and whole brain homogenate from these mice demonstrated high levels of DM20 proteins and low levels of PLP (Mastronardi, 1996; Barrese et al., 1998). Like the changes in MBP charge isomers, this profile was also characteristic of a developmentally immature myelin. Thus at adult ages, the presence of a less mature, less stable myelin
Chapter 3

characterized by changes in the two most abundant proteins in CNS myelin may be responsible for myelin breakdown. However, the reactions leading to these changes and how they contribute to myelin instability remains to be elucidated.

In the following chapters, results are presented from the search for the reactions responsible for the changes in PLP and DM20 protein levels and how this might contribute to myelin destabilization in the ND4 transgenic mouse. Since the ND4 mice each contain 70 copies of a DM20 cDNA transgene, a change at the genetic level, we began our study at the transcription factor level. This was followed by studies at the RNA level and the protein level. In chapter 3, PLP gene specific transcription complexes were assayed and found to be unchanged in ND4 transgenic mice. In chapter 4, DM20 RNA levels from the DM20 cDNA transgene were found to be in excess of both PLP and endogenous DM20 RNA levels. However, the level and stability of PLP gene transcripts were not affected by the abundance of transgene DM20 RNA. In chapter 5, PLP and DM20 protein isolated from ND4 transgenic mice were shown to be hyper fatty acylated. The findings from chapters 3 and 4 suggested that the defect responsible for myelin instability in the ND4 mice was due to a post-transcriptional event. This event could be the change in the post-translational modification of PLP and DM20 protein observed in chapter 5. The presence of hyperacylated PLP and DM20 protein may potentially affect the stability of the lipid bilayer and may increase the susceptibility of myelin to degradation.
Chapter 3: Levels of Transcription Factors Specific to the PLP Gene
Promoter in Normal and ND4 Transgenic Mice

Introduction

In elucidating the mechanism of demyelination in the ND4 transgenic mouse, the objective was to identify the biochemical reactions that lead to disease. Previous studies on the ND4 transgenic mice suggested that demyelination in these animals was the result of a biochemical change. These mice were characterized by high levels of DM20 protein and low levels of PLP in whole brain homogenate (Mastronardi, 1996). In myelin, DM20 protein was barely detectable at one month of age but the levels increased thereafter with age. Conversely, PLP levels in myelin decreased with age (Barrese et al., 1998). The low amount of PLP suggested that the ND4 mice were unable to synthesize PLP as efficiently as normal mice or it was more rapidly degraded. Since the ND4 mouse line was generated by inserting a normal DM20 cDNA transgene under the control of a normal PLP promoter into a normal mouse background, a possible explanation was that the abundant number of transgenes (70 copies) were competing for transcription factors required by the PLP gene for PLP synthesis. Though competition for transcription factors may not be a common phenomenon, it has been shown to occur between several proteins. Both the CREB-binding protein (CBP), a cofactor for the cyclic-adenosine monophosphate response-element-binding (CREB) transcription factor, and a closely related protein, the adenovirus E1A-associated 300kDa protein (p300), have been shown to be competed for by the transcription factor AP-1 and nuclear receptor hormones (Kamei et al., 1996), AP-1 and JAK/STAT (Horvai et al., 1997), and nuclear hormone receptors and E2F (Costa et al., 1996). Competition has also been shown to occur between the genetic imprinting genes, the H19 gene and the insulin-2 (Ins-2) and insulin-like growth factor-2 gene (Igf-2) (Bartolomei and Tilghman, 1992; Bartolomei et al., 1993). Competition for two
transcription enhancer proteins between the H19 gene and the Ins-2 and Igf-2 genes prevents the expression of the latter two gene from the maternal chromosome (Leighton et al., 1995). As a result, both the Ins-2 and Igf-2 gene are expressed only from the paternal chromosome resulting in genetic imprinting. Thus competition for transcriptional proteins between the DM20 cDNA transgenes and the PLP gene could result in the decreased levels of PLP.

To determine if the transgenes sequestered transcription regulatory proteins, the levels of DNA-binding proteins in ND4 mice were measured by gel mobility shift assay. This was done by assaying DNA-binding proteins which recognize $^{32}$P-labeled oligonucleotide probes whose sequences correspond to transcriptionally active regions of the PLP promoter (Fig. 3.1). The probes used in this study corresponded with two of five sites found in the proximal region of the PLP promoter (Fig. 3.1). These sites lie within the 312 bp region upstream of the transcriptional start site that is required for maximal activity (Berndt et al., 1992; Cambi and Kamholz, 1994). The binding of protein complexes from brain nuclear extract to these sequences have previously been characterized by L Hudson's group and the MSA experiments described in the following chapter were based on the methods used in their experiments. One of these sequences (oligo-4) has been identified as a specific site for a glial cell-specific transcription factor MyTi-1 (myelin transcription factor-1) (Kim and Hudson, 1992).

Since most of the transcription factors which bind to the PLP promoter have not been identified and those that have been (MyTi-1) have not been well characterized, conclusions made regarding the levels of specific transcription factors are suggestive. Without knowing which specific factors make up the DNA binding complexes and their binding properties and sensitivity to extraction with different salt concentrations, the results regarding the changes in their levels may have different interpretations.
With this in mind, the levels of DNA binding complexes specific for transcriptionally active regions of the PLP promoter were measured and compared to examine if the DM20 transgenes sequestered transcription complexes from the PLP gene. One interpretation of the findings is provided which may provide insight into whether a defect resulting in the changes in PLP and DM20 protein in the ND4 transgenic mice occur at a transcriptional level.

Results

*Gel mobility shift assay of DNA binding proteins specific to regions of the PLP promoter*

To determine if the 70 copies of the DM20 transgene sequestered transcription factors specific to the PLP gene promoter, brain nuclear extract from 14 day old normal and ND4 transgenic mice was prepared and assayed by gel mobility shift assay as described in the Methods section. The experimental procedure was the same as those described by Berndt et. al. (Berndt et al., 1992). Mice at 14 days of age were used to obtain optimal gene transcription conditions since the onset of myelination and the peak period of PLP gene expression begins at this age in mice (Gardinier et al., 1986). DNA-binding proteins were detected using $^{32}$P-labelled oligonucleotide probes corresponding to transcriptionally active elements in the PLP promoter (Fig. 3.1b)(Berndt et al., 1992). The amount of protein bound to these sequences was quantified by measuring the intensity of radioactivity in each band using a phosphor screen.
**Figure 3.1: PLP promoter sequence.** A) Proximal PLP promoter sequence. Boxed sequences are protein binding sites. TATA box is underlined and an inverted CCAAT element is marked by the arrow; putative NF-1 binding site (TGGCTNNNAGCCAA) in site 1. B) Sequences from the PLP promoter used as oligonucleotide probes from gel mobility shift assays. (Berndt et al., 1992)
DNA/protein complexes formed in brain nuclear extract from normal mice using oligo-1 probe

When brain nuclear extract from normal mice was assayed with oligonucleotide 1 (oligo-1) (Fig. 3.1b), two bands were observed in autoradiograms of the gel (Fig. 3.2b). These were referred to as oligo-1 binding factor 1 and 2 (BF₁-1 and BF₁-2). A large intense band near the bottom of the autoradiogram was from excess unbound labelled probe (Free probe). Neither BF₁-1 nor BF₁-2 were formed when either brain nuclear extract or labelled oligo-1 were excluded from the reaction mixture (Fig. 3.2b, lanes 1 and 2). This indicated that the bands arise from the binding of brain nuclear proteins to oligo-1. With increasing amount of protein, the intensity of both complexes increased and leveled off with 1.5 μg of total protein (Fig. 3.2a).

Of the two complexes formed, BF₁-1 was shown to have more specific binding for the oligo-1 probe than BF₁-2. This was determined by competition with the preincubation of unlabelled oligo-1 probe (Fig. 3.3). In this assay, the intensity of both complexes on the autoradiogram decreased with increasing amounts of unlabelled probe (Fig. 3.3b). The decrease in band intensity was more dramatic for BF₁-1 than for BF₁-2 (Fig. 3.3a). With 50 fold excess of unlabelled probe, BF₁-1 was virtually eliminated whereas BF₁-2 was decreased by only 1/10 (Fig. 3.3a). BF₁-2 was reduced to half with 100 fold excess of unlabelled probe. Thus, the protein (s) in the BF₁-1 complex bound more specifically to oligo-1 than those that formed BF₁-2. The specific components of BF₁-1 and BF₁-2 were not characterized nor were they reported in the literature. Although this sequence appears to contain a binding site for the transcription factor NF-1 (nuclear factor-1), the presence of this protein in the binding complexes has not been confirmed. Whether BF₁-1 and BF₁-2 are the same complex (two copies versus one copy of the same complex) or different complexes remains to be determined.
Figure 3.2: Gel mobility shift assay of brain nuclear extract (0.42 M KCl) from normal mice using oligo-1 probe. A. Saturation curves of BF₁-1 (■) and BF₁-2 (○) with increasing amounts of nuclear extract. B. Autoradiogram of gel mobility shift assay: lane 1, Oligo-1 probe alone; lane 2, without labelled oligo-1 probe; lane 3-6, with increasing amounts of nuclear extract (0.5μg, 1μg, 1.5μg and 2μg total protein).
Figure 3.3: Competitive mobility shift assay of brain nuclear extract (0.42M KCl) from normal mice using oligo-1 probe. A. Competitive decrease in BF$_1$-1 (■) and BF$_1$-2 (○) complexes with increasing amounts of unlabelled oligo-1 probe. B. Autoradiogram of competitive gel mobility shift assay: lane 1, Oligo-1 probe alone; lane 2, without labelled oligo-1 probe; lane 3, without unlabelled probe; lanes 4-6, with increasing amounts of unlabelled probe (10 fold, 50 fold, and 100 fold excess respectively).
Levels of DNA binding proteins specific for oligo-1 in ND4 mice

With 70 copies of the DM20 cDNA transgene, a reduction in the levels of PLP specific transcription factors is expected if transcription factors were sequestered by the transgenes. This was studied by assaying brain nuclear extract from 14 day old ND4 mice with oligo-1 as described in the Methods. The same DNA/protein complexes (BF₁-1 and BF₁-2) observed with brain nuclear extract from normal mice were resolved with brain nuclear extract from ND4 mice (Fig. 3.4b). Upon measuring and comparing the intensities of these bands, the level of both BF₁-1 and BF₁-2 were found to be the same in normal and ND4 transgenic (Fig. 3.4a). This suggested that the regulatory proteins specific to oligo-1 were not sequestered by the transgenes in the ND4 mice.

Comparison of the amount of DNA-binding proteins in brain nuclear extract prepared with buffer containing low salt and high salt concentrations

The objective of these experiments was to detect changes in the amount of unbound transcription factors, proteins that at any one time were not associated with DNA. Since the preparation of brain nuclear extracts employed 0.42 M KCl, the extraction of nuclear proteins at this salt concentration may have removed the total of both DNA bound and unbound proteins. Assuming that the factor NF-1 is present in the complexes formed using the oligo 1 probe, binding of this protein to specific DNA sites in an affinity column has been shown to dissociate at concentrations between 0.3M to 0.4M KCl but not at 0.1M KCl (Courtois et al., 1988). Thus preparation of nuclear extracts with 0.42 M KCl could potentially release all bound NF-1 proteins. To address this, nuclear extracts were prepared using a low salt concentration (0.1 M KCl). Since tight DNA binding proteins generally require salt extraction concentration of 2M or greater while loose-binding proteins are extracted using 0.3M NaCl (Campbell and Addey, 1979), bound DNA-binding proteins
should remain associated with DNA at this low salt concentration (0.1M). Proteins from this low salt extract were assayed and compared with the results using high salt extract. Results from gel mobility shift assays using the low salt extracts and oligo 1 probe showed that the amount of both BF1-1 and BF1-2 were similar to the amount measured in the high salt extracts (Fig. 3.5). Since the amount of both BF1-1 and BF1-2 were similar in low salt extracts from normal and transgenic animals, this suggested that the transgenes did not sequester transcription factors which bind to the oligo-1 sequence in the ND4 mice.

*Levels of DNA binding proteins specific for oligo-4 in ND4 mice*

Similar studies using a second sequence from the PLP promoter, oligo-4 (Fig. 3.1) were performed by Jodie Burton, a project student under my direction. This sequence was shown to be the binding site of the glial cell-specific transcription factor MyTI-1 (Kim and Hudson, 1992). Upon incubation of brain nuclear extract with $^{32}$P-labelled oligo-4 probe, five DNA/protein complexes were resolved in a mobility shift gel (Fig. 3.6). These were named oligo-4 binding factors 1, 2, 3, 4 and 5 (BF4-1, BF4-2, BF4-3, BF4-4 and BF4-5 respectively). Of the three most intense bands, BF4-3 was the most abundant while both BF4-2 and BF4-4 were present in lower quantities (Fig. 3.6). The appearance of these bands have been previously shown to occur upon incubation of oligo 4 with rat brain nuclear extract, extract from glial cell lines, and from cultured oligodendrocytes (Berndt et al., 1992; Kim and Hudson, 1992).

Competition for binding with unlabelled oligo-4 demonstrated that the complexes BF4-2, BF4-3 and BF4-4 were specific for oligo-4 (Fig. 3.7). This was also observed by Kim and Hudson (Kim and Hudson, 1992). In their experiment, MyTI-1 was expressed in bacterial cells transfected with a construct containing the human MyTI-1 cDNA. Using cell extracts containing the MyTI-1 protein, they found that competition with 100 fold excess unlabelled oligo-4 probe eliminated these three bands (Kim and Hudson,
Figure 3.4: DNA binding factors specific for oligo-1 probe in normal and transgenic mice. A) The amount of both binding factors BF₁-1 and BF₁-2 in brain nuclear extract (0.42M KCl) from 14 days old normal and transgenic mice. B) Autoradiogram comparing BF₁-1 and BF₁-2 between normal and transgenic samples. Lane 1, oligo-1 probe alone; Lane 2, normal; Lane 3, transgenic; □ BF₁-1; □ BF₁-2
These three bands corresponded with BF4-2, BF4-3 and BF4-4 in our experiment (Fig. 3.7). At the time these experiments were performed, the expression and characterization of MyTi-1 gene in mouse had not been well studied or cloned. Only a human cDNA for MyTi-1 was cloned. Since mouse brain nuclear extracts were used in our experiments, any differences in the protein interactions and complex formations by the mouse and human MyTi-1 protein prevents us from using the human clone as a control.

Figure 3.5: Comparison of the amount of binding proteins specific to oligo-1 probe in brain nuclear extract prepared with buffer containing 0.1M KCl and 0.42M KCl. Gel mobility shift assays were performed using brain nuclear extracts from normal and transgenic mice with buffer containing 0.1M KCl or 0.42M KCl. Using $^{32}$P-labelled oligo-1 as a probe, two bands were detected on an autoradiogram. The intensities were measured and normalized to the total intensity in each lane.

\( \text{\textbullet BF}_1-1, \quad \square \text{BF}_1-2. \) (Error bars, standard deviation 3 samples)
In our experiment, competition with increasing amounts of unlabelled oligo-4 demonstrated a gradual decrease in BF₄-2, BF₄-3 and BF₄-4. With the addition of 100 fold excess unlabelled probe, BF₄-2 and BF₄-4 were eliminated while trace amounts of BF₄-3 remained. This finding may be accounted for by the abundance of this complex in the assay. A greater amount of unlabelled probe may be required to completely eliminate this band. Regardless, these three complexes (BF₄-2, BF₄-3 and BF₄-4) have been shown to be formed by the binding of proteins specific for the sequence in the oligo-4 probe. One of these proteins may be the glial-cell specific transcription factor MyTi-1 (Kim and Hudson, 1992).

Levels of DNA binding proteins specific for oligo-4 in ND4 mice

The DNA/protein complexes formed with oligo-4 were examined using brain nuclear extract from ND4 mice. The number of bands and the changes that occurred with increasing amounts of extract was identical to the pattern observed with samples from normal mice. When the levels of these complexes were compared between normal and transgenic samples, the same concern regarding the salt extraction was addressed by preparing extract with buffer of lower salt concentrations (0.1M KCl). Since the ionic strength of MyTi-1 binding has not yet been characterized, a conclusion cannot be made regarding changes in the specific levels of MyTi-1. Thus changes in the levels of complexes formed were only considered. When the intensities of the complexes formed using oligo 4 were compared between samples from normal and ND4 mice, no difference was detected (Fig. 3.8). This suggests that the levels of complexes specific to oligo-4 were unaffected by the high number of DM20 transgenes and that changes in the amount of transcription factors did not account for the low amounts of PLP found in the ND4 mice.
Figure 3.6: Gel mobility shift assay of brain nuclear extract (0.42M KCl) from normal and ND4 transgenic mice with oligo-4 probe. Five bands (BF₄-1, BF₄-2, BF₄-3, BF₄-4 and BF₄-5) were observed in each lane. Lanes 1-4 contain brain nuclear extract from normal mice in increasing amount (2-15 μg of protein); Lanes 5-8 contain brain nuclear extract from ND4 transgenic mice in increasing amount (2-15 μg of protein); Lane 9 contains probe alone. (By J. Burton)
**Figure 3.7**: Competitive gel mobility shift assay of brain nuclear extract (0.42M KCl) from normal and ND4 transgenic mice with oligo-4 probe. Three bands (BF4-2, BF4-3, BF4-4) were observed in each lane. Lane 1, brain nuclear extract from normal mice without unlabeled oligo-4 probe; lanes 2-4, brain nuclear extract from normal mice with increasing amounts of unlabeled oligo-4 probe (10 fold, 50 fold, 100 fold molar excess, respectively); lane 5, brain nuclear extract from ND4 transgenic mice without unlabeled oligo-4 probe; lanes 6-8, brain nuclear extract from ND4 transgenic mice with increasing amounts of unlabeled oligo-4 probe (10 fold, 50 fold, 100 fold molar excess, respectively).
Figure 3.8: Intensity of three DNA/protein complexes specific for oligo-4 probe. BF4-2 ( ), BF4-3 ( ), BF4-4 ( )
Summary

The aim of this chapter was to determine if a decrease in the amount of available transcription factors was responsible for the decrease in the amount of PLP present in ND4 mice. This was addressed by measuring the levels of DNA/binding proteins complexes specific to transcriptionally active regions of the PLP promoter in brain nuclear extracts from normal and ND4 transgenic mice. The objective of the experiments was to detect changes in the amount of complexes. If the DM20 transgenes sequestered transcription factors, the expected result would be a decrease in the level of complexes.

The results presented in this chapter showed that several complexes were formed with both oligonucleotide probes. Some of these complexes were more specific for their respective sequences than others, for example, BF1-1 was more specific to oligo-1 than BF1-2. These differences may be important for the regulation of transcription. The amount of DNA-binding proteins specific for either of the oligonucleotide sequences was unchanged by the high copy number of the transgenes and they did not differ between normal and ND4 transgenic mice.

Since factors that bind specifically to the sites examined (sites 1 and 4, Fig. 3.1) have not been completely identified and well characterized, conclusions cannot be made regarding the levels of individual proteins. Furthermore, without knowing the binding properties of specific factors, the interpretation of our results may be difficult. As a result, we can only comment on the levels of bound complexes. The finding that the levels of complexes were unchanged between normal and transgenic animals can be interpreted in different ways. This result may be explained by the presence of excess transcription factors specific to the oligonucleotide probes such that no difference would be observed if competition were to occur, or by an artifact of the extraction method. Since no change was observed in the levels of complexes formed using low salt extracts (0.1M KCl), a
concentration well below the salt concentration at which NF-1 (a putative factor that binds to the PLP promoter) dissociate from its DNA binding site and at which loosely bound transcription factors dissociate from DNA (Campbell and Addey, 1979), it is possible that the levels of complexes measured reflects the amount of unbound protein. Although the sensitivity to salt concentration will depend on the specific transcription factors, DNA tight-binding proteins can only be extracted with 2M salt concentrations or greater and loose-binding proteins are extracted with 0.3M salt concentration (Campbell and Addey, 1979). Thus DNA bound proteins, tight and loosely attached, should remain associated in 0.1M KCl buffer. Nevertheless, further experiments will be needed to test the levels of bound and unbound protein in the different salt extractions. This can be examined by comparing the amount of protein in the salt extract with the amount of protein that remains bound to DNA in preparations using both low and high salt. Another approach could be to estimate the amount of DNA binding proteins in each extract by titrating with the oligonucleotide probes.

A third interpretation of the unchanged levels of specific complexes in transgenic mice could be a lack of competition for transcription complexes between the transgenes and the PLP gene, if the specific proteins are limiting. A lack of competition for transcription activating complexes would indicate that the changes in PLP and DM20 protein levels in the ND4 transgenic mice does not arise from differences in transcription. Further studies will be required to demonstrate whether transcriptional competition occurs between the DM20 transgene and the PLP gene in the ND4 transgenic mouse. On the basis of the examination of two of five transcriptionally active regions in the PLP promoter, one of which is recognized by a glial cell-specific transcription factor (MyTi-1), the decrease in PLP biosynthesis could not be explained by the levels of transcription activating complexes.
Chapter 4: Developmental Expression and Stability of PLP and DM20 RNA in Normal and ND4 Transgenic Mice

Introduction

In the previous chapter, it was demonstrated that the levels of transcription complexes were not reduced by the presence of 70 copies of the DM20 transgene in ND4 mice and that the low PLP amounts could not be explained by sequestering of transcription factors. To determine the reason behind the low amount of PLP and high amount of DM20 protein found in adult ND4 mice, further studies on RNA expression and stability were performed since this represented the next important level in protein synthesis.

In this chapter, the developmental expression of PLP RNA, endogenous DM20 RNA and transgene derived DM20 RNA was examined in both normal and ND4 mice. The amount of PLP RNA was compared with the amount of DM20 RNA to determine if the relative RNA levels reflected the change in the protein levels i.e. low PLP, high DM20. If the changes in protein levels were the result of alterations at the RNA level, one would expect a decrease in the amount of PLP RNA and an increase in the amount of DM20 RNA. This would indicate that the defect resulting in demyelinating disease in the ND4 mice occurred during RNA expression or RNA degradation.

As with PLP RNA, endogenous DM20 RNA was also thought to be reduced. Since very little DM20 protein was found in myelin at 1 month of age (Barrese, 1996), it was suggested that the protein found in myelin was translated from endogenous DM20 mRNA. It was proposed that the majority of DM20 protein was synthesized from the DM20 transgenes and that very little DM20 was made from the PLP gene. Although the DM20 protein produced from either the PLP gene or the transgene are identical, the RNA that encode for the two DM20s can be differentiated by size. Unfortunately, the endogenous DM20 RNA cannot be detected directly without detecting PLP RNA. Thus,
the expression levels on endogenous DM20 RNA can only be inferred by comparing the combined levels of PLP and DM20 RNA with the levels of PLP RNA alone.

The levels of PLP gene transcripts may be reduced by an increase in the rate of PLP RNA decay. As a result, PLP and DM20 protein derived from the PLP gene will also be reduced. This may explain the low amounts of PLP protein observed in transgenic mice and the low amount of DM20 protein found in myelin from young ND4 mice. Changes in the rate of decay was examined by measuring the half-life of PLP and endogenous DM20 RNA and transgene DM20 RNA.

The findings from these experiments are presented in this chapter and helped to define the mechanism of demyelination in the ND4 mice.

Results

Analysis of RNA from mouse brain

Total RNA from the brain of normal and ND4 transgenic mice ranging in age from 5 days to 8 months was extracted and analyzed by Northern blot as described in the Method section. Using either a PLP cDNA (pMD14) to detect PLP and DM20 RNA together or a PLP specific probe (ΔPLP) to detect PLP RNA only, RNA of different sizes from the PLP gene were found at 3.2 kb (the major species) and 2.4 kb (the minor species) and at 1.5 kb (Fig. 4.1a,b). PLP and endogenous DM20 RNA cannot be distinguished by size on and agarose gel. Therefore, these bands represent both PLP and DM20 when probed with pMD14. The intensity of both 3.2 and 2.4 kb bands were taken into account in determining the amount of RNA from the PLP gene. These values were normalized to the intensity of cyclophilin mRNA detected in each corresponding lane with a cyclophilin cDNA. The RNA from the DM20 transgene was detectable only with the pMD14 probe (Fig. 4.1 a,b). Although RNA was detected at the expected size (1.7 kb), a minor species was also found at 1.25 kb. This is thought to be due to an alternative poly-adenylation site
Figure 4.1: Autoradiograms of Northern blots of PLP, endogenous DM20 and transgene DM20 RNA expression in normal and ND4 transgenic mice. RNA samples were isolated from the brain of normal and ND4 mice ranging in age from 5 days to 8 months of age. Sample in each lane is identified in the autoradiogram. Blot a, probed with pMD14 (PLP cDNA) and cyclophilin cDNA; Blot b, probed with PLP specific probe (ΔPLP) and cyclophilin cDNA. N, normal sample; T, Transgenic sample; M, RNA markers.
within the transgene (Simons-Johnson et al., 1995). The intensities of both RNA species were used to determine the expression levels of RNA from the transgene.

*Developmental expression of PLP RNA in normal and ND4 transgenic mice*

Expression of PLP RNA was examined to determine if the RNA levels reflected the decrease in PLP levels found in both whole brain homogenate (Mastronardi, 1996) and myelin (Barrese, 1996). In normal mice, PLP RNA rose sharply from 5 days to 14 days and decreased gradually through 7 months of age (Fig. 4.2a). The levels peaked at 14 days of age which corresponded with the onset of myelination in mouse. This result is in agreement with findings reported in the literature which showed that the expression of PLP RNA reaches a peak at 14 days of age, remains high till 25 days of age and then declines thereafter to 20-30% of maximal levels in the mouse (Gardinier et al., 1986; Campagnoni and Macklin, 1988; Gardinier and Macklin, 1988; Shiota et al., 1989; Shiota et al., 1991). In the ND4 transgenic mice, the pattern of PLP RNA expression was similar to that found in normal mice. The RNA rose sharply between 5 and 14 days of age, peaked at 14 days and decreased gradually thereafter (Fig. 4.2a). The relative level of PLP RNA was identical between age matched normal and transgenic mice. Since PLP RNA levels were unchanged in ND4 mice at all ages, low PLP in both whole brain homogenate and in myelin cannot be explained by decreased transcript levels. This suggests that the low amount of PLP may be due to either increased turnover of PLP RNA or a defect at a post-transcriptional level.

*Developmental expression of DM20 RNA in normal and ND4 transgenic mice*

DM20 protein was found to be increased 4 fold in both whole brain homogenate (Mastronardi, 1996) and in myelin from adult ND4 mice (Barrese, 1996). However, the levels of DM20 protein in myelin from young ND4 mice was very low (Barrese, 1996).
Figure 4.2: Developmental expression of PLP gene transcripts and transgene transcripts. The amount of mRNA was determined by the Northern blot method. All bands were normalized to levels of cyclophilin mRNA. 

a: PLP mRNA expression profile,
b: PLP+Endogenous DM20 mRNA expression profile,
c: Transgene mRNA expression profile. Normal mice, □, Transgenic mice, ○. (Errors = Standard deviation from 3 separate blots)
Since DM20 RNA can be made from both the PLP gene and the DM20 transgene, the expression of DM20 RNA from the PLP gene was examined to determine if its expression is correspondingly increased or decreased due to expression of the transgene. Since DM20 RNA cannot be directly measured without detecting PLP RNA, insight into developmental expression pattern of DM20 RNA from the PLP gene was obtained by comparing the combined expression levels of both PLP+DM20 RNAs (measured using a PLP cDNA (pMD14)) with levels of PLP RNA alone (measured using a PLP specific probe (ΔPLP)). When the combined levels of PLP+DM20 RNAs from the PLP gene was measured in normal mice, PLP+DM20 RNAs increased sharply from 5 days and peaked at 14 days of age. Thereafter, the RNA decreased rapidly and leveled off at 6 month of age at approximately a third of peak levels (Fig. 4.2b). Similarly, in ND4 mice, PLP+DM20 RNA rose sharply from 5 days and peaked at 14 days of age (Fig. 4.2b). These levels decreased rapidly at 1 month of age and leveled off to approximately one third of peak values. When the expression levels were compared between normal and transgenic animals, the amounts of PLP+DM20 RNAs were unchanged throughout development. Since the combined levels of PLP and DM20 RNAs were unchanged in transgenic animals and since the levels of PLP RNA alone, measured by a PLP RNA specific probe, were also unchanged in transgenic mice (Fig. 4.2a), these data suggest that the expression of DM20 RNA from the PLP gene was not affected by the presence or the expression of the DM20 transgene. Therefore, the low levels of DM20 protein in the myelin from young ND4 mice cannot be explained by a decrease in DM20 RNA.

*Developmental expression of the DM20 RNA from the DM20 transgene*

Since the expression of DM20 RNA from the PLP gene appears to be unchanged in the ND4 mice, the 4 fold increase in DM20 protein can be attributed to the expression of the DM20 transgene. When the developmental expression of DM20 RNA from the transgene
was measured, it was found to increase sharply from 5 days and to level off at a value approximately 3 fold higher than the combined levels of PLP and endogenous DM20 RNA (Fig. 4.2c). This was maintained through to 8 months of age and correlated well with the 4 fold increase in DM20 protein measured in whole brain homogenate (Mastronardi, 1996).

Thus, the increase in DM20 protein in ND4 mice can be accounted for by the expression of the DM20 transgene. However, this does not explain the low amount of DM20 protein found in myelin at early ages (Barrese, 1996). Since the change in the amount DM20 protein in myelin was not reflected in the expression of RNA transcripts, this further implied that a defect leading to demyelination in the ND4 mice occurs at a post-transcriptional level. Although the transcript levels measured by Northern blot were unchanged, an increase in RNA degradation may account for the reduction in protein levels observed in the ND4 mice.

Stability of RNA transcripts from the PLP gene and the DM20 transgene in normal and ND4 transgenic mice

The RNA levels measured by Northern blot analysis gives an indication of the steady state levels of PLP RNA and does not provide information about the turnover of the transcript. Although the total PLP RNA levels in ND4 mice were found to be unchanged, a faster rate of turnover for PLP RNA in the ND4 mice could result in reduced synthesis of protein and explain the low protein levels measured in brain homogenate. To determine if the decrease in the amount of PLP in the ND4 mice compared to normal mice of the same age was related to a decrease in RNA stability, the half-life of PLP and endogenous DM20 RNA and the DM20 transgene RNA in 3 month old mice was measured. Freshly dissected mouse brain was cut into quarters and then incubated in media without submerging the tissue as described in the Methods. Incubation flasks were vigorously agitated with shaking in a water bath and loosely capped to allow air to pass through to facilitate passive
Figure 4.3: Autoradiogram of Northern blot performed for RNA stability assay. RNA samples were isolated from the brains of normal and ND4 transgenic mice incubated in media for 0, 1, 2, 4, 6, 9, and 18 hours (See experimental methods). Each lane contains 10 μg of RNA. The blot was probed with radiolabeled PLP cDNA (pMD14). N, Normal sample; T, Transgenic sample; M, RNA markers.
Figure 4.4: Decay curve of PLP, endogenous DM20 and transgene DM20 RNA from normal and transgenic mouse brain. The natural log of RNA band intensity normalized to 18S rRNA was plotted against the time of incubation. The line of best fit was determined by linear regression. The slope of this line (the rate of decay) was calculated and used to determine half-life. –– PLP and endogenous DM20 RNA from normal mice, Slope = -0.115; –•– PLP and endogenous DM20 RNA from transgenic mice, Slope = -0.110; ––•– Transgene DM20 RNA from normal mice, Slope = -0.106. (error bars = standard deviation on three separate experiments)
**Figure 4.5**: Decay of GAPDH RNA from normal and ND4 transgenic mouse brain. The natural log of GAPDH band intensity normalized to 18S rRNA was plotted against the time of incubation. A line of best fit was determined by linear regression from which the slope (the rate of decay) was calculated. This value was used to calculate the half-life of GAPDH RNA. 

- - - - - GAPDH RNA from normal mouse brain. Slope = -0.071.

- - - - - GAPDH RNA from transgenic mouse brain. Slope = -0.070. (error bars= standard deviation on three separate experiments)
diffusion of oxygen into the tissue for the duration of the experiment. At different time points, RNA was extracted from each brain and analyzed by Northern blot. The intensity of each band was measured and graphed against time of incubation to determine the half-life (Fig. 4.3). The intensity of the bands within each lane were normalized to the intensity of 18S ribosomal RNA levels. The slope from the RNA decay curve (the rate of RNA decay) was used to calculate the half-life (Fig. 4.4 and Fig. 4.5).

The half-life of PLP and endogenous DM20 RNA in normal mice was 6 hrs (Table. 4.1) and that of PLP and endogenous DM20 RNA was found to be 6.3 hrs in transgenic mice indicating that the stability of PLP and endogenous DM20 RNAs in transgenic mice was unaffected. RNA from the transgene had a similar half-life of 6.5 hrs. As a control, the half-life of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was measured and found to be 9.7 and 9.9 hrs for normal and transgenic mice respectively (Table. 4.1). Since the half-life of PLP RNA in ND4 mice was normal, the changes in PLP and DM20 protein expression in myelin from transgenic mice cannot be accounted for by a decrease in RNA stability. This suggests that a defect resulting in the reduction in PLP protein occurs at a post-transcriptional level in the ND4 mice.

**TABLE 4.1: Half-Life of mRNA in normal and transgenic mouse brains**

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<th>Normal (hrs)</th>
<th>Transgenic (hrs)</th>
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<tbody>
<tr>
<td>PLP and endogenous DM20 mRNA</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Transgene DM20 mRNA</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>GAPDH mRNA</td>
<td>9.7</td>
<td>9.9</td>
</tr>
</tbody>
</table>

A concern was noted regarding whether the decay rates measured in the stability assays were a reflection of the natural turnover of the RNA or a reflection of tissue death.
This concern was considered when the experiment was being designed; however, the problem was later dismissed for several reasons. First, the experimental procedure used to determine PLP RNA half-life was based on the established organotypic culture system used by Johnson and Keeley (Johnson and Keeley, 1990). In this assay, oxygen was provided to the tissue by vigorously agitating cultures to facilitate passive diffusion. These researchers demonstrated that the decay rates were unchanged between cultures that were oxygenated with 95% O2 and cultures that were oxygenated by passive diffusion (Johnson et al., 1995). Second, the half-life measured for GAPDH (the internal control) in the experiments presented in this chapter were the same as those reported for GAPDH in both oxygenated and passively diffused cultures examined by Johnson et al. (Johnson and Keeley, 1990, Johnson, 1995 #558). Lastly, a half-life of 5 hours for PLP/DM20 RNA, similar to the values reported in this chapter, was measured by Mathisen et al. using a brain slice culture system (Mathisen et al., 1997). Thus, the rate of decay for RNA measured using the culture system and reported in this chapter does not appear to be a reflection of tissue death. Instead, the results represent the decay of RNA in brain tissue in this culture system.

Summary

In this chapter, the developmental expression of PLP RNA, endogenous DM20 RNA and transgene derived DM20 RNA was examined to determine if the changes observed at the protein level were reflected at the RNA level. The findings from the Northern blot analysis showed that the expression of both PLP RNA and endogenous DM20 RNA were normal in ND4 mice while transgene DM20 RNA was elevated 3 fold over RNA transcript levels from the PLP gene. These results demonstrated that the decrease in the amount of PLP found in whole brain homogenate from transgenic mice was not due to a reduction in PLP RNA nor was the low level of DM20 protein in myelin from
1 month old ND4 mice due to reduced amounts of endogenous DM20 RNA. In fact, the expression of endogenous DM20 RNA did not appear to be affected by the 3 fold increase in DM20 transgene expression. Since the high amounts of DM20 protein measured in whole brain homogenate corresponded with the high amounts of transgene DM20 RNA, the majority of the DM20 protein was probably translated from the transgene DM20 RNA. Therefore, the expression levels of RNA from the natural PLP gene was not affected by the transgene.

Although the RNA levels were normal, a decrease in RNA stability may result in reduced protein levels. This was examined by measuring the half-lives of PLP and endogenous DM20 RNA in ND4 mice. The results from RNA stability assays demonstrated that the half-life of PLP and endogenous DM20 RNAs from the PLP gene in ND4 mice was unchanged. PLP and endogenous DM20 RNA was just as stable in ND4 mice as it was in normal mice. Thus, the decrease in PLP protein levels was not due to a decrease in PLP RNA stability.

Although the rates of RNA synthesis were not measured in this study, the low amounts of PLP in whole brain homogenate of ND4 mice suggested that the transgenic mice were unable to synthesize PLP as efficiently as normal mice. The findings from this study demonstrated that the low PLP protein levels were not explained by changes at the RNA level. Neither the amounts of PLP RNA measured by Northern blot nor the half-life of PLP RNA were changed in the ND4 mice. Considering these results with the finding that the changes in the PLP and DM20 protein levels could not be explained at the transcription factor level (chapter 3), it was concluded that the biochemical reactions resulting in demyelinating disease in the ND4 mice occurred at a post-transcriptional level perhaps during translation or with post-translational modifications.
Chapter 5. Isolation and Characterization of PLP and DM20 from Normal and ND4 Transgenic Mouse Brain

Introduction

The biochemical reaction(s) that leads to demyelination in the ND4 transgenic mice was suggested by the findings presented in chapters 3 and 4 to occur at a post-transcriptional level since transcripts and RNA stability studies were the same in transgenic and normal animals. The myelin from adult ND4 transgenic mice was characterized by high levels of DM20 protein and low levels of PLP (Barrese, 1996) suggesting that a detailed study of the proteins represents the next step. Although the obvious experiment would be to examine the synthetic rates of PLP and DM20 proteins in ND4 mice, the long half-life (~28 days) and the highly insoluble nature of PLP and DM20 would make these experiments difficult to perform and to assess. Furthermore, since cultured primary oligodendrocytes would not survive long enough to allow measurement of the turnover rate of PLP and DM20, injection of animals with a radiolabelled amino acid would be required. However, these experiments would be difficult since a high percentage of animals often do not survive the injection procedure. Thus a large number of animals (normal and transgenic) would be required for this type of experiment which could be limited by the life span of the transgenic mice. For these reasons, the synthetic rates for PLP and DM20 were not assessed at this time. Instead, the PLP and DM20 proteins were examined for changes in post-translational modification. This alternative avenue was pursued due to an experiment performed by another student in the lab who demonstrated that the migration of PLP and DM20 in myelin separated on SDS-PAGE were slightly retarded (Mastronardi et al., 1996c). Thus changes in the post-translational modification of these proteins may provide insight into why the levels of PLP are reduced in the ND4 mouse and maybe into the mechanism of myelin destabilization.
Aside from the formation of disulfide bonds, fatty acylation is the only naturally occurring post-translational modification in PLP and DM20 known at this time. Palmitoylation, which was first found to occur in PLP and DM20 along with covalently bound stearate (Gagnon et al., 1971; Stoffyn and Folch-Pi, 1971), plays a role in the targeting of proteins such as G-proteins to membrane bilayers. In G-proteins, this modification of the C-terminal amino acid can affect their association and orientation with lipids or other proteins (Milligan et al., 1995; Morello and Bouvier, 1996). Fatty acylation of PLP and DM20 though not at the C-terminus may serve a similar role in the assembly and interactions with proteins and lipids in myelin.

Although high levels of DM20 protein were measured in the myelin from adult transgenic mice, very little DM20 protein was detected in the myelin from 1 month old transgenic mice (Barrese, 1996). In contrast, at 1 month of age, high levels of DM20 protein were measured in whole brain homogenate (Mastronardi, 1996). Similarly, PLP levels in transgenic mice plateaued by 2 months of age and remained at this level throughout adulthood (Mastronardi, 1996) well below the amounts found in normal adult mice. This suggested that not all of the PLP and DM20 proteins were assembled into myelin. It was postulated that the discrepancy between the levels of protein in whole brain homogenate and in myelin were due to changes which altered the assembly of PLP and DM20 into myelin. Since the primary structure of the proteins was unaltered compared to normal, changes in the amount of covalently bound fatty acids on PLP and DM20 might explain the low amounts in early myelin. Thus PLP and DM20 protein from 1 month old normal and ND4 transgenic mice was isolated and fractionated by ion-exchange chromatography on a CM-Tris acryl column using the method described by Helnyck et. al. (Helnyck et al., 1983). The amounts of covalently bound fatty acids on both PLP and DM20 were then quantitated by GC/MS.
Results

Analysis of PLP and DM20 in myelin from DM20 transgenic mice by Western blot

Change in post-translational modification of PLP and DM20 from transgenic mice was implicated by the results from an experiment performed by Dr. F. G. Mastronardi (Mastronardi et al., 1996c). In this experiment, myelin prepared from normal, heterozygous ND3a transgenic, homozygous ND3a transgenic, and ND4 transgenic mice was separated by SDS-PAGE and analyzed by Western blot using a PLP/DM20 specific antibody (CT-PLP/Ab105; reacts with residues 200-224 of PLP) (Fig. 5.1). Heterozygous ND3a animals contain 17 copies of DM20 cDNA, homozygous ND3a mice contain 34 copies of DM20 cDNA while the ND4 mice contain 70 copies of the same DM20 cDNA transgene. The migration of both PLP and DM20 were observed to be slowed in the heterozygous ND3a (Fig. 5.1, Lane 3), homozygous ND3a (Fig. 5.1, Lane 4) and ND4 mouse (Fig. 5.1, Lane 5) samples compared to normal mouse samples (Fig. 5.1, Lane 2). This result suggested that both PLP and DM20 were alternatively modified in DM20 transgenic animals. This change in modification appeared to be dose dependent since the shift in migration was greatest in the ND4 mouse sample compared with both ND3a animals and the shift in migration was greater in the homozygous ND3a mouse compared with the heterozygous ND3a sample. Since PLP and DM20 are naturally modified by covalently bound fatty acids, the nature of this modification was subsequently pursued by isolating PLP and DM20 from ND4 transgenic animals and measuring the amount of covalently bound fatty acids.
Figure 5.1: Western blot of myelin isolated from normal and DM20 transgenic mice. Myelin fractions were isolated from the brains of normal, heterozygous ND3a, homozygous ND3a, and ND4 transgenic mice by the method of Norton and Poduslo (1973). Myelin proteins were separated through a 10-20% Tricine linear gradient gel and transferred onto nitrocellulose for Western blot analysis. The blot was incubated with CT-PLP/Ab105 antibody which reacts with residues 200-224 of PLP. This antibody reacts to both PLP and DM20 protein (arrows). Lane 1: prestained molecular weight markers, Lane 2: normal mouse myelin, Lane 3: 12 month old heterozygous ND3a mouse myelin (17 copies of DM20 transgene), Lane 4: 10 month old homozygous ND3a mouse myelin (34 copies of DM20 transgene), Lane 5: 10 month old ND4 mouse myelin (70 copies of DM20 transgene). (Used with permission of M. G. Mastronardi, from Mastronardi et al., 1996c)
Isolation of PLP and DM20 from mouse brain

Twenty brains from 1 month old normal or transgenic mice were used to isolate PLP and DM20. Total brain proteolipids were prepared and delipidated as described in the Methods. Approximately 10 OD280nm units of total protein suspended in chloroform-methanol-water (4:4:1, v/v/v) were applied to a CM-Tris acryl column. The column was washed with chloroform-methanol-water (4:4:1, v/v/v) and DM20 and PLP were eluted with 0.01M and 0.05M solutions of ammonium acetate respectively in chloroform-methanol-water (4:4:1, v/v/v). Two ml fractions were collected.

A typical CM-Tris acryl column profile of total brain proteolipids (Fig. 5.3) had several minor peaks and a major peak which were collected within the void volume. These peaks included low molecular weight proteins and may be due to the presence of plasmolipin or other small unidentified proteolipids. The amounts of these low molecular weight proteins were small and they were not characterized. Elution of proteins from the column with increasing concentrations of ammonium acetate in chloroform-methanol-water (4:4:1, v/v/v) produced two major peaks. The first peak eluted with 0.01M ammonium acetate and the second with 0.05 M ammonium acetate. These peaks have been previously characterized and were shown to contain DM20 protein and PLP respectively in several studies (Helnyck et al., 1983).

The fractionation of DM20 and PLP on a CM-Tris acryl column was first described by Helnyck et al. (Helnyck et al., 1983). Separating purified bovine brain proteolipids by this method, these investigators obtained a similar elution profile as those presented here. By Western blot and by amino acid analysis, they demonstrated that the peak which eluted with 0.01 M ammonium acetate contained DM20 while the peak which eluted with 0.05 M ammonium acetate consisted of PLP. In a study by another student in our laboratory (Nancy Barrese), brain proteolipid isolated from mouse was separated using the same method described by Helnyck et al. (Helnyck et al., 1983). The elution profile from her
<table>
<thead>
<tr>
<th></th>
<th>nmoles</th>
<th>ngrams</th>
<th>Res/100</th>
<th>Res/mol</th>
<th>Expected Res/100 for DM20</th>
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<td>Asx(D+N)</td>
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<tr>
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</tr>
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<td>2.090</td>
<td>0.01</td>
<td>3.7</td>
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Total | 40.76  | 4226.67 | 100    | 0.25    |

*Figure 5.2: Amino acid analysis of peak 2 from a CM-Tris acryl column profile of proteolipids from normal mouse brain. Normal mouse brain proteolipid were extracted using chloroform:methanol:water (4:4:4) and separated on a CM-Tris acryl column as described in the Methods. Fractions eluting in a single peak with 0.01M ammonium acetate (peak 2) were pooled and the composition of this peak was analyzed by amino acid analysis. The results were compared with the theoretical amino acid composition of DM20 protein. (experiment performed by N. Barrese, amino acid analysis by the Biotechnology centre, Banting and Best institute, Toronto)*
Figure 5.3: CM-acryl column separation profile of total brain proteolipid from 1 month old normal mice. A total of 10 OD_{280nm} units of total brain proteolipids isolated from at least 20 mice was loaded onto the column. The column was washed with 25 ml of chloroform:methanol:water (4:4:1) and then eluted with a step gradient of 0.01 M ammonium acetate in chloroform:methanol:water (4:4:1) (25 ml) followed by 0.05 M ammonium acetate in chloroform:methanol:water (4:4:1) (50 ml). Each fraction was collected in 2 ml volumes and the absorbance at 280nm was measured on a spectrophotometer. The following fractions under each peak were pooled and analyzed: fractions 9 to 11 (peak 1), fractions 15 to 17 (peak 2), and fractions 40 to 43 (peak 3).

- - - - Abs 280 nm  - - - - Conc. Am. Ac. (M)
Figure 5.4: CM- acryl column separation profile of total brain proteolipid from 1 month old transgenic mice. A total of 10 OD_{280nm} units of total brain proteolipids isolated from at least 20 mice was loaded onto the column. The column was washed with 25 ml of chloroform:methanol:water (4:4:1) and then eluted with a step gradient of 0.01 M ammonium acetate in chloroform:methanol:water (4:4:1) (25 ml) followed by 0.05 M ammonium acetate in chloroform:methanol:water (4:4:1) (50 ml). Each fraction was collected in 2 ml volumes and the absorbance at 280nm was measured on a spectrophotometer. The following fractions under each peak were pooled and analyzed: fractions 10 to 12 (peak 1), fractions 15 to 17 (peak 2), and fractions 40 to 44 (peak 3).
columns demonstrated the elution of a peak with 0.01 M ammonium acetate in fractions 14-16 and another peak with 0.05 M ammonium acetate, in agreement with the findings reported by Helnyck et al. (Helnyck et al., 1983). Characterization of these peaks by Western blot using PLP specific antibodies confirmed the elution of DM20 in the first peak and PLP in the second peak. Also, the composition of the first peak (eluted with 0.01 M ammonium acetate) was analyzed by amino acid analysis and shown be consistent with the composition of DM20 (Fig. 5.2). The fractionation was further confirmed with pure brain PLP which was shown to elute in fractions 40-45.

The elution profile of the proteolipids isolated from normal and transgenic mice is shown in figure 5.3 and figure 5.4 respectively. The column profiles were basically similar except that the material eluting in fraction 10 was greatly increased in the transgenic sample compared to normal. Although the peaks collected from fractionation of purified proteolipid proteins and proteolipid proteins from mouse brain using this column system have previously been characterized (Helnyck et al., 1983) (N. Barrese, unpublished), small quantities from each peak collected from the columns represented by figure 5.3 (normal sample) and figure 5.4 (transgenic sample) were analyzed by SDS-PAGE and by Western blot. In the Western blots, two antibodies were employed to distinguish PLP from DM20: CT-PLP/Ab105 (detects both PLP and DM20) and ΔDM20 Ab (detects PLP only). Due to the hydrophobicity of PLP and DM20 and their extreme tendency to aggregate and precipitate, analysis of these proteins by SDS-PAGE and Western blot do not reflect the relative quantities of these proteins in the peaks. In some cases no protein was observed on the gel or blot probably due to loss of protein by precipitation. Protein from the fractions from normal mice (Fig. 5.3) were separated by SDS-PAGE (Fig. 5.5) and analyzed by Western blot (Fig. 5.6 a and b). On SDS-PAGE, peak 1 was resolved into several bands. One intensely stained band occurred at a high $M_r$ (Fig. 5.5, lane 3) while another band resolved below the position of PLP in myelin (Fig. 5.6, lane 6). These two bands and
Figure 5.5: SDS-PAGE of peaks collected from CM-tris acryl column of normal mouse brain extract. Protein in one O.D.280nm unit from each peak was ether precipitated and mixed in 2X SDS sample buffer as described in the Methods. Protein from the starting material was treated in the same manner. From each preparation 5μl was loaded onto the gel. Lane 1: prestain molecular weight markers, Lane 2: starting material, Lane 3: peak 1, Lane 4: peak 3, Lane 5: peak 2, Lane 6: normal mouse myelin.

Figure 5.6: Western blot of peaks collected from CM-tris acryl column of normal mouse brain extract. Sample were prepared and loaded as described for the SDS-PAGE gel. a) Western blot using CT PLP/Ab 105. Lane 1: peak 1, Lane 2: peak 3, Lane 3: peak 2, Lane 4: normal mouse myelin.
b) Western blot using ADM20 Ab. Lane 1: prestained molecular weight markers, Lane 2: peak starting material, Lane 3: normal mouse myelin, Lane 4: peak 1, Lane 5: peak 3, Lane 6: peak 2.
several others of higher $M_r$ reacted to the CT-PLP/Ab 105 antibody (Fig. 5.6a, lane 1) but not to the $\Delta$DM20 antibody (Fig. 5.6b, lane 4) indicated that peak 1 contained some DM20 protein. However, most of the DM20 seemed to be aggregated as the reactivity to CT-PLP/Ab 105 antibody occurred near the top of the gel. Some of the DM20 in the normal sample may have passed through the column with the wash as aggregates and thus may explain the detection of DM20 in peak 1. Peak 1 contained other protein as well as observed by a band of low $M_r$ which did not react with either of the two PLP antibodies (Fig 5.6a, lane 1; Fig 5.6 b, lane 4). This protein was not characterized. In the lane containing peak 2, very little protein was detected (Fig. 5.5, lane 6). No reactivity was observed with CT-PLP/Ab 105 antibody (Fig. 5.6a, lane 3) or with $\Delta$DM20 antibody (Fig. 5.6b, lane 6). Since DM20 is extremely hydrophobic and can easily precipitate out of solution, the lack of staining on SDS-PAGE and reactivity to PLP/DM20 specific antibodies on Western blot suggests that the protein in peak 2 was lost during the preparation of the sample. This peak was previously characterized by another student in the laboratory who demonstrated the presence of DM20 in this peak by amino acid analysis (Fig. 5.2). The proteins in peak 3 were very faint (Fig. 5.5, lane 4), probably also due to loss of some protein during sample preparation. However, on Western blots, a single sharp band with the same $M_r$ as PLP in myelin (Fig. 5.6a, lane 4; Fig. 5.6b, lane 3) reacted with both CT-PLP/Ab 105 (Fig. 5.6a, lane 2) and $\Delta$DM20 (Fig. 5.6b, lane 5) antibodies indicating that peak 3 contained PLP.

Similarly the peaks collected from the transgenic sample (Fig. 5.4) were analyzed by SDS-PAGE (Fig. 5.7) and Western blot (Fig. 5.8 a and b). As in peak 1 from the normal sample, two intensely stained bands were observed in peak 1 from the transgenic (Fig. 5.7, lane 2). The darker band migrated slightly below the position of PLP in myelin (Fig. 5.7, lane 5). However, these two bands did not react with the two PLP antibodies
Figure 5.7: SDS-PAGE of peaks collected from CM-tris acryl column of ND4 transgenic mouse brain extract. Protein in one O.D. 280nm unit from each peak was ether precipitated and mixed in 2X SDS sample buffer as described in the Methods. Protein from the starting material was treated in the same manner. From each preparation 5μl was loaded onto the gel.
Lane 1: starting material, Lane 2: peak 1, Lane 3: peak 2, Lane 4: peak 3, Lane 5: normal mouse myelin, Lane 6: prestain molecular weight markers.

Figure 5.8: Western blot of peaks collected from CM-tris acryl column of ND4 transgenic mouse brain extract. Sample were prepared and loaded as described for the SDS-PAGE gel. a) Western blot using CT PLP/ Ab 105. Lane 1: starting material, Lane 2: peak 1, Lane 3: peak 2, Lane 4: peak 3, Lane 5: normal mouse myelin, Lane 6: prestained molecular weight standards.
b) Western blot using ADM20 Ab. Lane 1: prestained molecular weight standards, Lane 2: normal mouse myelin, Lane 3: peak starting material, Lane 4: peak 1, Lane 5: peak 3, Lane 6: peak 2.
when analyzed by Western blot (Fig. 5.8a, lane 2 and Fig. 5.8b, lane 4) suggesting that these two bands may contain protein other than DM20 or PLP. This may also be true for the bands observed in pack 1 from the normal sample (Fig. 5.5, lane 3). Some reactivity, however, was observed near the top of the gel with CT-PLP/Ab 105 antibody. This suggests that DM20 eluted in this peak perhaps in aggregated form. Since no bands were resolved in peak 2 by SDS-PAGE (Fig. 5.7, lane 3) and no reactivity was observed by Western blot (Fig. 5.8a, lane 3 and Fig. 5.8b, lane 6), protein from peak 2 was probably lost during sample preparation due to aggregation and precipitation of DM20. Separation of peak 3 on SDS-PAGE resolved several bands most of which had low Mr (Fig. 5.7, lane 4). The most intensely stained band had a Mr similar to PLP in myelin (Fig. 5.7, lane 5). This band reacted with both CT-PLP/Ab 105 and ΔDM20 antibodies indicating that it contained PLP (Fig. 5.8a, lane 4 and Fig. 5.8b, lane 5). A band migrating slightly ahead of PLP reacted with CT-PLP/Ab 105 antibody (Fig. 5.8a, lane 4) but not with ΔDM20 antibody (Fig. 5.8b, lane 5). Thus peak 3 from the transgenic sample (Fig. 5.4) also contained some DM20. Some DM20 protein may have been retained on the column during elution due to increased aggregation with PLP in the transgenic sample. As a result, this may have increased the difficulty in separating these two protein and explain the co-elution of DM20 with PLP.

Although DM20 was not detected in peak 2 in either columns (Fig. 5.3 and 5.4) using SDS-PAGE and Western blot, this may be an artifact of sample preparation. The presence of DM20 in peak 2 was demonstrated by amino acid analysis, a more accurate analysis of this peak (Fig. 5.2). Protein prepared from peak 2 and peak 3 from both columns were analyzed for the levels of covalently bound fatty acids. PLP peak material from both columns was further purified using a preparative gel and PLP and DM20 were eluted from an excised gel slice as described in the Methods.
Analysis of covalently bound fatty acids on DM20 and PLP isolated from the brains of normal and ND4 transgenic mice

Each protein was hydrolyzed and derivatized to obtain the fatty acids for GC/MS as described in the Methods. This work was performed by Lawrence Fisher (Hospital for Sick Children, Toronto). In initial experiments, DM20 and PLP samples were analyzed in full spectrum on GS/MS and palmitate, stearate, and N-methyl glycin were identified by comparing the mass spectra to a library data base. Since the specific ion peaks were relatively small in the full spectra scan, which made quantitation difficult, subsequent samples were analyzed in the selected ion recording mode (SIR) which increased the sensitivity by 100 fold. In this recording mode, Palmitate, stearate, and N-methyl glycine were each identified by their retention time on gas chromatography and then by the most prominent ion specific to their respective mass spectrum. The abundance of this ion was quantitated relative to a known standard mix which was analyzed in the same mode on the same day. Since the samples were recorded in the SIR mode, the full mass spectrum of each species could not be recorded and are not reported here. Only the gas chromatograms noting the specific retention times for the specific m/z ion could be presented (Figs. 5.9 and 5.10). The purity of the samples and the specificity of the measurements was attributed to a single sharp peaks eluting from the gas column, the specific retention time which corresponded with the elution time of known standards on the same column, and the selection of the most prominent ion specific to the species from its mass spectrum.

The abundance of palmitate and stearate were measured in PLP and DM20 protein from normal and ND4 mice. The data from this experiment are shown in Table 5.1. Since the amount of protein recovered in each peak from the column was small, protein assays were not performed to determine concentration amounts. Instead, the values for each fatty acid were normalized to the OD_{280nm} units in each sample. When DM20 was analyzed, the amount of palmitate increased from 1.93 nmoles/OD_{280nm} in the normal sample to
3.18 nmoles/OD\textsubscript{280\text{nm}} in the transgenic sample. A transgenic to normal ratio of 1.6 indicated that the amount of covalently bound palmitate was almost doubled in DM20 from transgenic mice. Similarly, stearate increased from 0.35 nmoles/OD\textsubscript{280\text{nm}} in the normal sample to 0.96 nmoles/OD\textsubscript{280\text{nm}} in the transgenic sample. A transgenic to normal ratio of 2.7 for this fatty acid indicated that the amount of covalently bound stearate was elevated three fold in the transgenic sample.

**TABLE 5.1. Fatty acid quantitation of DM20 and PLP from normal and transgenic mice (nmoles/OD\textsubscript{280\text{nm}} units).** Values were obtained by normalizing GC/MS peak areas for the individual fatty acid to the peak area of the internal standard and then dividing by the OD\textsubscript{280\text{nm}} units in the sample.

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<th>Protein</th>
<th>Fatty acid</th>
<th>Normal</th>
<th>Transgenic</th>
<th>Ratio (T/N)</th>
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<td>DM20</td>
<td>Palmitate</td>
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<tr>
<td></td>
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<td>0.96</td>
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<td></td>
<td>Stearate</td>
<td>1.82</td>
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\textsuperscript{a} Two determinations on independent proteins samples (Includes data from experiment by N. Barrese)

\textsuperscript{b} PLP, proteolipid protein

When PLP was measured, larger changes were found (Fig. 5.9). Palmitate increased from 1.51 nmoles/OD\textsubscript{280\text{nm}} to 9.31 nmoles/OD\textsubscript{280\text{nm}} in normal and transgenic samples respectively. This gave a transgenic to normal ratio of 6.2. Stearate increased from 1.82 nmoles/OD\textsubscript{280\text{nm}} in normal samples to 11.6 nmoles/OD\textsubscript{280\text{nm}} in transgenic samples. Its transgenic to normal ratio was similar to that calculated for palmitate, 6.4. These results show that the amount of covalently bound palmitate and stearate was
Figure 5.9: GC/MS chromatograms of covalently bound fatty acids in PLP isolated from normal (a) and ND4 transgenic (b) mice. Palmitic acid (PA) and stearic acid (SA) in normal and transgenic PLP samples are each shown in individual chromatograms with the peak area and the retention times (respectively) above. Pentadecanoic acid (PD) was used as an internal standard for each run. The total abundance for the species and the species identifying m/z value is noted on the upper right hand corner for each spectrum.
Figure 5.10: GC/MS chromatograms of N-methyl glycine (sarcosine) in PLP isolated from ND4 transgenic (A) and normal (B) mice. The presence and abundance of N-methyl glycine in each PLP sample was measured and plotted in the chromatograms. N-methyl glycine eluted from the column with a retention time of 9.88 minutes as demonstrated by the chromatogram of pure N-methyl glycine (C). The amount of N-methyl glycine, shown above the peak, was more abundant in PLP from ND4 mice compared to normal animals.
increased in each proteolipid from transgenic mice and that PLP was more heavily acylated than DM20. Other fatty acids such as oleate which represented 15% of the total and trace amounts of linoleate and palmitoleate were found but not quantitated.

In addition to covalently bound fatty acids, N-methyl glycine (sarcosine) was detected in PLP and DM20 (not reported here) from both the normal and transgenic samples (Fig. 5.10). Since this modification could only arise from the methylation of the N-terminal glycine of PLP and DM20, the detection of N-methyl glycine suggests that PLP and DM20 was N-terminally blocked. The levels of N-methyl glycine on PLP from normal animals was low (Fig. 5.10B) which would explain why this modification had not been previously detected on PLP and DM20 and reported in the literature. In comparison, N-methyl glycine on PLP from transgenic animals was increased approximately 19 fold (Table 5.2). Therefore, the combined effects of increased fatty acylation and N-terminal methylation would make these proteolipids more hydrophobic and consequently could affect their assembly or orientation within the lipid bilayer.

**TABLE 5.2. Quantitation of N-methyl glycine (sarcosine) on PLP from normal and transgenic mice (nmoles/OD$_{280}$nm units).** Values for sample A and B (Fig. 5.10) were obtained by normalizing GC/MS peak areas for N-methyl glycine to the peak area of the internal standard (pentadecanoic acid, not shown) and then dividing by the OD$_{280}$nm units in each sample.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Normal</th>
<th>Transgenic</th>
<th>Ratio (T/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>0.04</td>
<td>0.76</td>
<td>19.0</td>
</tr>
</tbody>
</table>
Summary

In this chapter, fatty acylation of DM20 and PLP was examined in both normal and ND4 transgenic mice. To isolate DM20 from PLP, total brain proteolipids were fractionated on a CM-Tris acryl column and eluted with different concentrations of ammonium acetate solution. Using proteolipids from both normal and transgenic mice, DM20 eluted in a single peak with 0.01 M ammonium acetate while PLP eluted with 0.05 M ammonium acetate. Analysis of fractionated DM20 and PLP by GC/MS demonstrated an increase in the amount of covalently bound fatty acids in protein from transgenic mice. Compared with DM20 from normal mice, the amount of palmitate covalently bound to DM20 was increased approximately 2 fold in transgenic mice while stearate was increased 3 fold. When PLP was examined, the difference in the amount of covalently bound fatty acids was even greater. The amount of palmitate and stearate were both increased over 6 fold. The absence of detection for DM20 in SDS-PAGE and Western blots (Fig. 5.7 and Fig. 5.8) and the co-elution of DM20 with PLP in the transgenic sample (Fig. 5.8) may be explained by the increase in covalently bound fatty acids on both DM20 and PLP from transgenic mice. Hyperacylation of these extremely hydrophobic proteins may enhance and strengthen the aggregation of DM20 proteins and promote precipitation. Similarly, the interactions of hyperacylated DM20 and PLP may be stronger and more difficult to separate in the transgenic sample. The increase in covalently bound fatty acids on PLP and DM20 from ND4 transgenic mice was supported by the finding that the migration of these proteins in SDS-PAGE was retarded in transgenic samples (Fig. 5.1). Hyperacylation of PLP and DM20 may be dependent on the transgene dose since PLP and DM20 in myelin from transgenic mice containing ~35 copies of DM20 cDNA transgene migrated faster than PLP and DM20 in myelin from ND4 mice containing 70 copies of transgene but slower than PLP and DM20 from normal mice (Fig. 5.1). Interestingly, post-translational modification of MBP (the amount of deiminated MBP) was also shown to be dose dependent in DM20.
transgenic mice (Mastronardi et al., 1996c). Thus, the presence of the transgene or the overexpression of DM20 RNA or protein may be responsible for the changes in post-translational modification of the major proteins in CNS myelin.

PLP and DM20 were also shown to be naturally methylated at the N-terminus in low amounts but greatly increased in the transgenic mice (20 fold). This modification has been found to occur in several different proteins each involved in interacting with other proteins within macromolecular complexes, for example, ribosomes, nucleosomes myofibrils etc. (Stock et al., 1987). Although the effects of N-terminal methylation on proteins are speculative and a role in protein interactions remains to be demonstrated, the increase in N-methyl glycine on PLP and DM20 may serve a similar purpose by increasing the interactions of these proteins within myelin. Since the N-terminus of PLP and DM20 is believed to be closely associated with the lipids of the myelin bilayer through palmitoylation at nearby cysteine residues, an increase in N-terminal methylation can increase this association. Together, these changes in post-translational modification can potentially alter the structure of PLP and DM20 in myelin resulting in a loss of myelin order and an increase in susceptibility to attack and disruption.

Concluding remarks

One of the objectives of this thesis was to elucidate biochemical reactions that leads to demyelination in the ND4 transgenic mice. In this chapter, both PLP and DM20 isolated from the brain of transgenic mice were shown to be hyperacylated. It was postulated that this change may alter the nature of PLP in myelin and in turn affect the stability of the bilayer.

PLP naturally contains 2-3 mols of covalently bound fatty acids per mol of protein (Gagnon et al., 1971; Stoffyn and Folch-Pi, 1971). These fatty acids are primarily palmitate, stearate, and oleate (Stoffyn and Folch-Pi, 1971) but their function on PLP is yet
to be defined. Since covalently bound fatty acids have been shown to target, to anchor, and to aid interactions of other proteins such as G-proteins and Rhodopsin with lipids and other proteins (Milligan et al., 1995; Morello and Bouvier, 1996), the covalently bound fatty acids of PLP may aid in its function in myelin by altering its orientation and interactions within the lipid bilayer. Although the role(s) of PLP in myelin remains ambiguous, one of the functions implied from studies of PLP deficient mice was the maintenance of the structural spacing in myelin (Boison and Stoffel, 1994; Rosenbluth et al., 1996; Klugmann et al., 1997). Thus changes in the acylation of PLP may affect its interactions with lipids and consequently the structure of myelin.

Another property of PLP is its potential to act as an ion channel. With four transmembrane domains, PLP structurally resembles a group of different ion channels such as the ryanodine, nAChR, GluR, Gaba receptors to name a few (Takeshima et al., 1989; Unwin, 1989; Knapp, 1996). Functionally, PLP was shown to induce conductance across an artificial lipid bilayer measured by $^{22}\text{Na}^+$ influx (Helnyck et al., 1983). PLP was also postulated to have Ca$^{2+}$ gating properties by resisting Ca$^{2+}$ influx into liposomes in an ATP-dependent manner (Diaz et al., 1990). Thus PLP could act as an ion channel.

If hyperacylation of PLP changes the structure and orientation of PLP in a lipid bilayer, the channeling properties of PLP may also be affected. As a result, it can be speculated that the ion distribution could be disrupted within myelin. One possible effect could be the inhibition of Ca$^{2+}$ gating by PLP which may increase Ca$^{2+}$ levels within myelin. Since PAD has been shown to be calcium activated (Rogers et al., 1977), an increase in Ca$^{2+}$ levels could enhance PAD activity and the modification of arginyl residue to citrullinyl residues in MBP. Consequently, myelin may become destabilized and more susceptible to attack. Thus changes in the acylation of PLP may affect its interactions with lipids and consequently its structure in myelin. This could lead to changes in its actions in maintaining the structure of compact myelin and its actions as an ion channel.
Since we believe that demyelination is primarily a myelin related event, we can now propose one mechanism for destabilizing myelin in transgenic mice (Fig. 5.11). Based on studies of the ND4 transgenic mice (a model for demyelinating disease), this mechanism involves changes in the post-translational modification of myelin proteins. The increase in the fatty acylation of PLP described in this thesis is suggested as the initial event followed by deimination of arginyl residues and digestion of MBP, all of which result in membrane destabilization and breakdown.

As a result of a genetic lesion in the ND4 mice, the first process may be the synthesis of an altered protein such as hyperacylated PLP. The accompanying disorganization of the bilayer (Fig. 5.11 (1)) relieves enzymes present in myelin such as PAD, cathepsin D, and MMPs from the constraints of a compact lipid bilayer. Although I have not generated evidence for this in the thesis, another graduate student in the laboratory is currently doing so.

With the removal of these constraints, an increase in PAD activity resulting in the deimination of arginines into citrullines in MBP could follow (Fig. 5.11 (2)). The generation of MBP-C8 and the subsequent loss of net positive charge reduces the ability of MBP to interact with the lipid bilayer which will further compromise the compaction and stability of the myelin bilayer (Fig. 5.11 (3)). Increased levels of MBP-C8 has been shown to occur in the myelin isolated from the brain of both MS patients and ND4 mice (Moscarello et al., 1994; Mastronardi et al., 1996b). Citrulline content in MBP also correlated with disease severity (Wood et al., 1996). A recent finding demonstrating fragmentation of multilayered lipid vesicles by MBP-Cit18 suggests that vacuolation of the myelin bilayers can be facilitated by increased citrulline content in MBP (Boggs et al., 1999) through which MBP can be shed from myelin (Fig. 5.11 (4)). Therefore, an increase in PAD activity can potentially enhance the release of MBP from myelin which can activate extracellular responses ie. astrogliosis toward demyelination.
The activation of astrocytes by MBP has been shown in our lab to occur in astrocytoma cells \textit{in vitro} (Owh et al., 1997). Uptake of MBP by astrocyte was shown to occur through binding with the surface receptor molecule CD44. In response, astrocytes up regulate CD44 synthesis (Owh et al., 1997), a homing molecule found on both astrocytes and oligodendrocytes (Moretto et al., 1993) that can facilitate the homing of astrocytes to myelin (Fig. 5.11 (5)). In addition, astrocytes secrete matrix metalloproteinases (MMPs) (Gottschall and Yu, 1995) that can degrade components of blood brain barrier (BBB) (Fig. 5.11 (8)) (Rosenburg et al., 1992; Leppert et al., 1995; Rosenberg et al., 1995; Leppert et al., 1996; Stuve et al., 1996; Clement et al., 1997) which allow sensitized lymphocytes and macrophages to invade into the brain (Fig. 5.11 (9)). Through these reactions, the release of MBP from myelin can contribute to myelin breakdown.

Another result of a less compact lipid bilayer could be increased digestion of MBP by matrix metalloproteinases (MMPs) (Chandry et al., 1989; Chandry and Glynn, 1990) and cathepsin D (Azaryan et al., 1983) present in myelin (Fig. 5.11 (6)). These enzymes have been shown to generate encephalitogenic peptides from MBP (Brostoff et al., 1974; Whitaker and Seyer, 1979; Gijbels et al., 1993; Chandler et al., 1995) which are capable of sensitizing lymphocytes and stimulating the immune component in demyelination (Fig. 5.11 (7)).

Thus changes in the post-translational modification of myelin proteins could be the initiating event which destabilize the myelin lipid bilayer and result in the release of myelin components which stimulate the extracellular responses towards myelin degradation. The processes in this mechanism include: i) disruption in the organization of the lipid bilayer in myelin by hyper fatty acylated PLP/DM20 and the removal of constraints imposed on enzymes by a compact myelin; ii) the increase in citrullinated MBP by PAD; iii) induction of astrogliosis and targeting of astrocytes to myelin; iv) induction of an immune response
Figure 5.11: Processes involved in demyelinating disease.
1) Disruption of the bilayer. 2) Deimination of MBP by PAD. 3) Further disorganization of bilayer. 4) Shedding of MBP. 5) Activation of astrocytes and the induction of astrogliosis and homing to myelin. 6) Digestion of MBP by cathepsin D and MMP generating MBP peptides. 7) Sensitization of lymphocytes. 8) MMP digests BBB allowing sensitized lymphocytes into the brain. 9) Autoimmune phase begins.
through the generation of MBP encephalitogenic peptides by cathepsin D and MMPs and increased permeability of the BBB. Therefore, these processes are viable targets for drugs in the treatment of disease. Ideally, effective intervention by a drug to halt the process of demyelination must target several or all of these processes.
Part 2: Effect of Paclitaxel as a Therapeutic Agent in the Treatment of Demyelinating Disease

Although Multiple Sclerosis (MS) is the most common demyelinating disease in adult humans (Ludwin, 1995), the etiology remains unknown. Four recent studies designed to locate susceptibility loci in patients suffering from MS has provided some insight into a genetic origin of the disease (Ebers et al., 1996; Haines et al., 1996; Kuokkanen et al., 1996; Sawcer et al., 1996). In these studies no single major site was identified; however, multiple genetic loci were found to be mildly associated with disease. It was postulated that instead of one genetic locus, several sites each with an effect are required for MS. This complexity suggests that the pathogenesis of disease must also involve several interconnecting processes which lead to demyelination.

Based on the studies of autopsy material and cerebrospinal fluid obtained from MS patients and on studies of the ND4 transgenic mice (a model of demyelinating disease), a series of processes involved in the disease have been postulated in the previous chapter (Chapter 5) (fig. 5.11). In approaching an effective treatment for disease, a therapeutic agent must target these processes. The different actions of paclitaxel (Taxol) suggests that it may be a good candidate drug. Paclitaxel is traditionally known to inhibit cell proliferation by binding to microtubules and preventing their disassembly (Schiff et al., 1979; Horwitz, 1992; Rao et al., 1994). In our lab, we have shown that paclitaxel inhibits the activity of PAD on benzoylarginine ethyl ester (BAEE) and MBP in vitro (Pritzker and Moscarello, 1998). Paclitaxel has been shown to inhibit the activator-protein (AP-1) dependent transcription of MMPs (stromelysin and collagenase) in cultured chondrocytes (Hui et al., 1998). Lastly, Whitaker et. al. found that paclitaxel can inhibit the uptake of $^3$H-thymidine by MBP activated lymphocytes (personal communication). Thus paclitaxel can i) inhibit MMP expression, ii) inhibit the activity of PAD, and iii) inhibit proliferation
Figure 6.1. Targets for Paclitaxel action. 1) Inhibition of PAD activity. 2) Inhibition of MMP expression. 3) Inhibition of astrocyte and lymphocyte proliferation.
of astrocytes and lymphocytes (Fig. 6.1). In this manner, paclitaxel can target multiple processes in the pathogenesis of demyelinating disease.

In this section, we report the findings from the treatment of ND4 transgenic mice with paclitaxel. In chapter 6, we showed that paclitaxel i) reduced progression of the disease and the severity of clinical signs and ii) decreased the number of astrocytes in transgenic mice. In chapter 7, the expression of the matrix metalloproteinase (MMP), stromelysin-1, was found to correlate with the onset of disease in the ND4 mouse. Although stromelysin RNA levels were not reduced upon treatment of ND4 mice with paclitaxel, expression or activation of other MMPs may be affected. In chapter 8, evidence of remyelination is presented, perhaps the result of inhibition of proliferating astrocytes. Based on these findings, it was concluded that paclitaxel was an effective drug for the treatment of demyelinating disease.
Chapter 6. Attenuation of Clinical Signs and the Inhibition of Proliferating Astrocytes in ND4 Transgenic Mice by Paclitaxel

Introduction

In this chapter, the effect of paclitaxel on the clinical progression of demyelinating disease in the ND4 transgenic mice is presented. Through its different properties, paclitaxel was thought to reduce the severity and the progression of disease. Of importance is its antimitotic effect on proliferating cells. By targeting and binding to the N-terminus of β-tubulin (Horwitz, 1992; Rao et al., 1994), paclitaxel promotes microtubule assembly and prevents disassembly of microtubule polymers (Schiff et al., 1979). This binding is thought to involve the taxane ring in the structure of paclitaxel (Fig. 1.15) (Nicholov et al., 1997). Paclitaxel may inhibit proliferating astrocytes by the same mechanism. Since astrogliosis is a prominent feature of demyelination, the effect of paclitaxel on astrogliosis was studied in the ND4 mouse. Inhibition of astrogliosis could reduce the severity of the attack on oligodendrocytes and myelin and remove the inhibitory effect of astrocytes on remyelination. This inhibitory effect will be discussed in greater detail in a later chapter. In this chapter, the effect of paclitaxel on astrocytes in ND4 mice was examined by immunocytochemical staining for GFAP (a marker for astrocytes), electron microscopy of astrocyte ultrastructure, and immunoslot blot quantitation of GFAP in whole brain homogenate. These data are presented in the following sections.

Results

*Paclitaxel attenuates the clinical progression of disease in ND4 transgenic mice*

Paclitaxel was injected intraperitoneally into normal and ND4 transgenic mice at a dose of 20 mg paclitaxel/ kg body weight as described in the Methods. These mice were treated either at the onset of clinical signs (3 to 3.5 months of age) (10 to 15 mice) or prior
to the onset of clinical signs (2 to 2.5 months of age) (Four animals). Normal age matched mice were also injected with paclitaxel in control experiments. No side affects were observed in these animals. Each mouse was injected once each week for four weeks and were clinically scored every second day. The clinical criteria (outlined in Fig. 6.2) were scored on a four point scale as described in the Methods. The scores for each animal were summed each week and plotted against age to obtain a graph of their clinical progression (Fig. 6.2).

The sum of scores per week for ND4 mice treated with PBS increased sharply from 3 months to 4.5 months of age. By 4.5 months, the score reached a plateau of approximately 55 to 60 and remained at this level throughout 6 months of age (Fig. 6.2a). In ND4 transgenic mice treated with paclitaxel at the onset of clinical signs, the clinical scores increased gradually with age. By 6 months of age, the clinical score appeared to level off at approximately half the value (25 to 30) of age matched transgenic control mice (Fig. 6.2b). The scores were reduced even further in ND4 transgenic mice treated with paclitaxel before the onset of clinical signs i.e. at 2 months (Fig. 6.2c). By 5.5 to 6 months of age, the scores from this set of animals reached a plateau at approximately 1/4 (10 to 15) the maximum value of transgenic control mice. Furthermore, the onset of signs occurred later in these mice compared with both transgenic mice treated at the onset of signs and the transgenic controls. These results indicate that paclitaxel can reduced the clinical severity of disease and delay the time of onset of signs in transgenic mice. More importantly, paclitaxel remains effective long after injections have been stopped. Thus the attenuation of clinical signs by paclitaxel long after its injection makes this drug a good candidate for the treatment of demyelinating disease.
Figure 6.2: Aggregate clinical scores of paclitaxel treated and untreated transgenic mice with progression of disease. Mice were scored three times a week every week from the beginning of the experiment till time of death. Sum of scores from each week were determined and plotted against age. Each point is an average of sums from three or more animals. a) ■ Aggregate scores from untreated transgenic mice. b) ● Aggregate scores from transgenic mice treated at the onset of signs (10-15 mice). c) ○ Aggregate scores from transgenic mice treated before the onset of signs (4 mice). (error bars = standard deviation)

Inhibition of astrocytes by paclitaxel

Transgenic mice were treated with paclitaxel at the onset of clinical signs with the same regimen and dosage described in the Methods. Animals were sacrificed two months after the last injection at 6 months of age. Brain sections were examined under light
microscopy for immunocytochemical staining of glial fibrillary acidic protein (GFAP, a marker for astrocytes) and under electron microscopy for ultrastructural organization of astrocytes. To determine the extent of astrogliosis, the amount of GFAP was measured in brain homogenates by immunoslot blot and the number of GFAP stained cells were counted under light microscopy as described in Methods.

Immunocytochemical staining for GFAP in brain sections

Brain sections from 6 month old normal and transgenic mice were stained for glial fibrillary acidic protein (GFAP), a marker for astrocytes (Fig. 6.3). White matter tracts in sections from normal mouse brain had very little cell staining. Stained cells were stellate in shape with well defined proximal and distal processes (Fig. 6.3a, arrowheads). In comparison, white matter tracts from ND4 mouse brain sections were heavily stained showing dense clusters of astrocytes (Fig. 6.3b, arrowheads). Both the intensity and the number of cells were increased compared with the section from normal mouse (Fig. 6.3a). Unlike the section from untreated transgenic mice, clusters of astrocytes were not visible in stained sections from paclitaxel treated transgenic animals. The amount of staining in brain sections from these mice was similar to the degree of staining in the normal sample (Fig. 6.3c). However, the majority of staining within each cell occurred in the proximal processes and in the cell body (Fig 6.3c, arrowheads). The cell bodies were rounded which suggested that these cells may be dying. The distal processes were not stained but a faint halo around each astrocyte was observed which could be remnants of the distal processes. This halo may be the result of GFAP released from a dying cell. Thus paclitaxel targeted proliferating astrocytes in ND4 mice.
Figure 6.3: Light microscopy of GFAP stained brain sections. Sections of brain white matter taken from 6 month old mice. Astrocytes in each section are identified by arrowheads. a) Section from untreated normal animal. b) Section from untreated transgenic animal. c) Section from Paclitaxel treated transgenic animal. Bar= 100μm.
Quantitation of the number of GFAP stained cells and the amount of GFAP in whole brain homogenate

To obtain a quantitative measure of the effect of paclitaxel on astrocytes in ND4 mice, the number of GFAP stained cells and the amount of GFAP present was determined. Using the GFAP stained sections from the 6 month old mice (described above), the number of stained cells in paclitaxel treated ND4 mice was 10.3±2.2 astrocytes/mm² (Table 6.1). This was comparable to the number of cells observed in normal mice (7.9±2.7 astrocytes/mm²) and lower than the number of stained cells in untreated transgenic mice (16.3±3.7 astrocytes/mm²) (Table 6.1). GFAP levels in the brain homogenate from these mice were measured by immunoslot blot. The amount of GFAP in paclitaxel treated transgenic mice was 13.8 ng/µg total protein. This was similar to the amount measured in normal mice (12.8 ng/µg total protein) (Table 6.1). The amount in untreated ND4 mice was about 3 fold greater than that of treated ND4 mice indicating that the astrocytes are in fact reduced in paclitaxel treated ND4 mice. The decrease in the number of GFAP stained

\[TABLE\ 6.1.\ \text{Amount\ of\ GFAP\ in\ whole\ brain\ homogenate\ and\ number\ of\ GFAP\ stained\ cells\ in\ mouse\ brain\ sections}\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>GFAP (ng)</th>
<th>GFAP (ng/µg homogenate protein)</th>
<th>Number of astrocytes/mm²*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.64±0.02</td>
<td>12.8</td>
<td>7.9±2.7</td>
</tr>
<tr>
<td>Transgenic</td>
<td>1.80±0.10</td>
<td>36.0</td>
<td>16.8±3.7</td>
</tr>
<tr>
<td>Transgenic (Paclitaxel)</td>
<td>0.69±0.05</td>
<td>13.8</td>
<td>10.3±2.2</td>
</tr>
</tbody>
</table>

* Minimum 100 fields/ treatment group. P>0.05

Cell counts were performed by C. A. Ackerley (Hospital for Sick Children, dept. Pathology, Toronto).
cells and the amount of GFAP in whole brain homogenate indicates that paclitaxel inhibits proliferating astrocytes. This was consistent with the ultrastructural study showing destruction of astrocytes in ND4 mice (see below).

_Ultrastructure of astrocytes in paclitaxel treated ND4 mice_

Electron microscopy of a brain section from an untreated normal mouse shows an astrocyte near a blood vessel in Figure 6.4a. The filaments within this astrocyte were well organized (Fig. 6.4a, *). A comparative area from a normal mouse treated with paclitaxel is shown in Figure 6.4b. The filaments in this cell (Fig. 6.4b, *) were as well organized as those seen in astrocytes from the untreated normal mice. However, some lysosomes were present in these cells (Fig. 6.4b, arrowheads). Thus paclitaxel does not have any adverse effects on filaments in normal astrocytes.

The effect of paclitaxel on astrocytes in ND4 mice is shown in Figure 6.5. The electron micrograph of white matter from a 6 month old untreated transgenic mouse shows an astrocyte in association with an endothelial cell (EN) near a blood vessel (Fig. 6.5a). The intermediate filaments within this astrocyte were well organized (Fig. 6.5a, *). Other structures were also well defined such as mitochondria (M) and some ingested myelin (Mye). In paclitaxel treated transgenic mice, the microtubule filaments within astrocytes were disrupted and disorganized (Fig. 6.5b, *). With the exception of these filaments and some mitochondria, the cytoplasm in these cells was mostly void. In Figure 6.5b, an empty space (arrows) observed near the blood vessel suggested that an astrocyte process may have once occupied this space. Furthermore, the nucleus appeared to be rounded while the chromatin was condensed and accumulated at the periphery of the nucleus. Astrocytes within white matter tracts away from blood vessels had similar features (Fig. 6.5c). Thus paclitaxel affects the ultrastructure of astrocytes in ND4 mice.
Figure 6.4: Electron microscopy of sections through normal brain white matter. Sections were taken from 6 month old normal mice. Filaments within an astrocyte are identified by the asterisk (*). Endothelial cells are marked by EN.
a) Section from a normal untreated animal showing an astrocyte surrounding an endothelial cell of a blood vessel.
b) Section from a Paclitaxel treated normal animal showing an astrocyte surrounding an endothelial cell of a blood vessel. Lysosomes within this astrocyte are marked by arrowheads. Bar= 1 μm.
(EM by C. A. Ackerley, Hospital for Sick Children, Dept. of Pathology, Toronto)
Figure 6.5: Electron microscopy of sections through white matter from transgenic mice. Sections were taken from 6 month old transgenic mice. Filaments within the astrocytes are marked by the asterisk (*). Mitochondria are identified by (M). a) Section from an untreated transgenic animal showing a filamentous astrocyte in association with an endothelial cell (EN) of a blood vessel. Myelin is identified as (Mye). b) Section from a Paclitaxel treated transgenic animal. An astrocyte near an endothelial cell (EN) of a blood vessel. The filaments within the astrocyte are sparse and disorganized. The nucleus (N) appears rounded with peripheral chromatin. An empty space surrounding the endothelial cell is marked by arrows. c) Section from Paclitaxel treated transgenic animal in an area away from blood vessels. The ultrastructure of this astrocyte is the same as the cells observed near blood vessels (b). Bar=1 μm. (EM by C.A. Ackerley, Hospital for Sick Children, Dept. of Pathology, Toronto)
Summary

The effects of paclitaxel on the clinical progression of disease in the ND4 mice was examined in this chapter. Treatment of transgenic mice after the onset of clinical signs was shown to reduce the clinical scores by half. The severity of clinical signs were further reduced if treatment began prior to the onset of clinical signs. The scores in these mice remained much lower than both untreated transgenic mice and transgenic mice treated at the onset of signs. In both sets of animals treated with paclitaxel, the reduction in score was maintained two months after treatment was stopped indicating the paclitaxel has long term effects. Furthermore, the progression of disease was attenuated. This was more noticeable in mice treated before the onset of signs whose clinical scores did not rise above 10 until after 5 months of age. Thus paclitaxel was effective in reducing both the severity and the progression of disease in ND4 transgenic mice. The anti-mitotic properties of paclitaxel on astrocytes may in part be responsible for these finding.

Astrogliosis has been shown to occur in ND4 mice by immunocytochemical staining for GFAP (Mastronardi et al., 1993). Since astrocytes in normal mice were not adversely affected by paclitaxel, this drug specifically targeted the proliferating astrocytes in ND4 mice. This was supported by the reduction in GFAP stained cells and the amount of GFAP in whole brain homogenate from ND4 mice. Ultrastructural analysis by EM showed disruption of cellular organization within astrocytes. Rounded nuclei with peripheral chromatin and loss of distal processes suggested these astrocytes were dying. Based on these findings, it was concluded that paclitaxel inhibits proliferation and promotes astrocyte cell death in the ND4 mice. By inhibiting astrogliosis, in the ND4 transgenic mice, paclitaxel could permit remyelination and recovery to occur.
Chapter 7: Expression of Matrix Metalloproteinases (MMPs) in the ND4 Transgenic Mice

Introduction

Matrix metalloproteinases (MMPs), a family of Zn$^{2+}$ dependent endoproteases that digest components of the extracellular matrix are involved in tissue remodeling and cell motility (Woessner, 1994; Murphy, 1995). To date, 19 MMPs have been identified (Table 7.1). Most of these proteinases reside and function extracellularly while several have been found to be membrane bound. These enzymes are tightly regulated at many levels. One of these levels occur during transcription at which synthesis of MMP transcript may be coordinated through the action of the transcription factor AP-1 (activator protein-1). With the exception of gelatinase-A (MMP-2), the promoter region of all MMPs contain a binding site for AP-1 (Fig. 7.1) (Benbow and Brinckerhoff, 1997). Synthesized and maintained as inactive proenzymes, MMPs are also regulated through an inhibitory propeptide at the N-terminus which when released activates the enzyme (Nagase, 1997). This is thought to occur through autocleavage or cleavage by other MMPs (Nagase, 1997). Activation of MMP can also occur through a cascade mechanism. Plasmin, released from plasminogen by the action of urokinase plasminogen activator (uPA), can activate stromelysin (MMP-3) which can in turn activate other MMPs (Fig. 7.2) (Yong et al., 1998). In effect, this cascade amplifies MMP activity allowing for quick responses during cell remodeling or motility. To complement this, activated MMPs are readily controlled by natural inhibitors of MMPs, the tissue specific inhibitors of matrix metalloproteinases (TIMPs) (Gomez et al., 1997). Four members have so far been described each expressed in different tissues and with specificities to different MMPs. TIMPs bind to and inhibit both the activation of pro-MMPs and the activity of activated MMPs (Nagase, 1997).
Table 7.1: Family of Matrix metalloproteinases (MMPs) and their substrates. 
(From Nagase, 1997)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MMP no.</th>
<th>Matrix substrates or functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>Collagens I, II, III, VII, and X, gelatins, aggrecan, link protein</td>
</tr>
<tr>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>Collagens I, II, and III, aggrecan, link protein</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>MMP-13</td>
<td>Collagens I, II, III</td>
</tr>
<tr>
<td>Collagenase 4 (Xenopus)</td>
<td>MMP-18</td>
<td>Collagen I</td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>Gelatins, collagens I, IV, V, VII, X, and XI, fibronectin, laminin, aggrecan, elastin, large tenascin C, vitronectin, β-amyloid protein precursor</td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>Gelatins, collagens IV, V, XIV, aggrecan, elastin, entactin, vitronectin</td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin 1</td>
<td>MMP-3</td>
<td>Aggrecan, gelatins, fibronectin, laminin, collagens III, IV, IX, and X, large tenascin-C, vitronectin</td>
</tr>
<tr>
<td>Stromelysin 2</td>
<td>MMP-10</td>
<td>Aggrecan, fibronectin, collagen IV</td>
</tr>
<tr>
<td><strong>Membrane-type MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Collagens I, II, III, fibronectin, laminin-1, vitronectin, dermatan sulfate proteoglycan: activates proMMP-2 and proMMP-13</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>MMP-15</td>
<td>Not known</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>MMP-16</td>
<td>Activates proMMP-2</td>
</tr>
<tr>
<td>MT4-MMP</td>
<td>MMP-17</td>
<td>Not known</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>Aggrecan, fibronectin, laminin, gelatins, collagen IV, elastin, entactin, small tenascin-C, vitronectin</td>
</tr>
<tr>
<td>Stromelysin 3</td>
<td>MMP-11</td>
<td>Weak activity on fibronectin, laminin, collagen IV, aggrecan, gelatins</td>
</tr>
<tr>
<td>Metalloelastase</td>
<td>MMP-12</td>
<td>elastin</td>
</tr>
<tr>
<td><strong>Unnamed</strong></td>
<td>MMP-19</td>
<td>Not known</td>
</tr>
</tbody>
</table>
Figure 7.1: MMP promoters showing the location of cis-acting elements known to participate in transcription. Promoters from various MMPs from different species are shown. All but one MMP (MMP-2, gelatinase A) contain an AP-1 binding site. Binding sites: TATA, Tata box; AP-1, activator protein-1; AP-2, activator protein-2; PEA 3, polyomavirus enhancer A-binding protein-3; TIE, TGF-β inhibitory element; CG, SP-1 binding site; SPRE, stromelysin-1 PDGF responsive element binding protein; NF-κβ, nuclear factor κβ. (Adapted from Benbow and Brinckerhoff, 1997).
Figure 7.2: Cascade of matrix metalloproteinase (MMP) activation.
The co-ordinated activation of several MMPs is initiated by the formation of plasmin from plasminogen by the action of urokinase plasminogen activator (uPA). This is anchored to the cell membrane by its receptor, uPAR. Plasmin can activate stromelysin-1 (MMP-3) and gelatinase B (MMP-9) directly and other MMPs such as collagenase-1 (MMP-1) in a cascade-like manner. *(Adapted from Yong et. al., 1998).*
MMPs have been implicated in myelin degradation and may mediate an immune response through the release of encephalitogenic peptides by digesting myelin basic protein (Gijbels et al., 1993; Chandler et al., 1995). Several MMPs (gelatinase B (MMP-9), stromelysin (MMP-3) and collagenase (MMP-1)) have been detected in plaque regions and the cerebrospinal fluid (CSF) obtained from MS patients (Gijbels et al., 1992; Gijbels and Steinman, 1994a; Maeda and Sobel, 1996). More specifically, gelatinase B has been localized to astrocytes and monocytes near capillaries in MS lesions (Gijbels et al., 1994b; Cuzner et al., 1996) and its activity has been found to be increased in CSF of both MS patients and EAE (Gijbels et al., 1992; Gijbels et al., 1993). These MMPs have been shown to produce encephalitogenic peptides by digesting MBP (Gijbels et al., 1993; Chandler et al., 1995).

Chandry et. al. have demonstrated that a 60 kDa MMP found in myelin is capable of producing encephalitogenic peptides from myelin basic protein in vitro (Chandry et al., 1989; Chandry and Gynn, 1990). Induction of an inflammatory response in lymphocytes by these fragments may stimulate the release of cytokines which can activate additional MMPs (Ries and Petrides, 1995). Since MMPs are also known to degrade the components of the blood brain barrier (BBB), digestion of the BBB by MMPs may allow myelin components to pass through and to activate lymphocytes. In response, these activated cells can infiltrate the brain by further weakening the BBB through the action of MMPs. Transmigration of T-cells in vitro has been shown to be MMP dependent by several groups (Rosenburg et al., 1992; Leppert et al., 1995; Rosenberg et al., 1995; Leppert et al., 1996; Stuve et al., 1996; Clement et al., 1997). Through the release of encephalitogenic peptides from MBP and the digestion of components forming the BBB, MMP activity may facilitate the autoimmune response in demyelinating disease.

In our transgenic model (Mastronardi et al., 1993), astrogliosis represents an important cellular response to destabilized myelin since these cells engulf and ingest myelin
membrane pieces. The mobility of astrocytes through the tissue depends on digestion of extracellular matrix by Matrix Metalloproteinases (MMPs). Since MMPs have been implicated in the degradative processes in demyelinating disease, I studied MMP expression and have implicated one major MMP in the process of disease in the ND4 mice.

Using the ND4 transgenic mice as a model of demyelinating disease, the expression levels of MMPs during the course of disease was examined by Northern blots. Expression of c-Jun and c-Fos, which dimerize to form AP-1, and TIMP-1 was also examined to determine if the regulatory pathways for MMPs were also changed in the ND4 mice. In chapters 6, paclitaxel was shown to attenuate demyelinating disease in the ND4 mice. Since paclitaxel has been shown to regulate the expression of MMPs through the inhibition of AP-1 activity in cultured chondrocytes (Hui et al., 1998), by extension paclitaxel may have a similar effect on the expression of MMPs in ND4 mice. Thus MMP expression was also examined in paclitaxel treated ND4 mice.

Results

Developmental expression of stromelysin-1 (MMP-3) in normal and transgenic mice

A Northern blot containing RNA from normal and ND4 transgenic mice ranging from 5 days to 8 months of age was probed with a stromelysin-1 cDNA probe as described in the Methods. Stromelysin RNA was barely detectable in normal samples (Fig. 7.3A). The intensity increased slightly with age but the value of the bands remained low (Fig. 7.3B). In transgenic mice, stromelysin-1 RNA was low at early ages but increased sharply by 1 month of age (Fig. 7.3A and B). These amounts reached a plateau after 3 months of age at approximately 5 fold higher than normal (Fig. 7.3B). Thus, the expression of stromelysin-1 RNA was upregulated and this increase corresponded with onset of disease in ND4 mice.
**Figure 7.3:** Developmental expression of stromelysin RNA in mouse brain. A) Northern blot of the developmental expression of stromelysin-1 RNA in normal and transgenic mice. B) Plot of band intensities of Stromelysin-1 RNA. ◇ normal mice; □ transgenic mice.
Expression of c-Jun and c-Fos RNA in normal and transgenic mice

Since the activation of AP-1, by heterodimeric complex of c-Jun and c-Fos proteins, is required for MMP production (Benbow and Brinckerhoff, 1997), expression of c-Jun and c-Fos was also examined in the ND4 mice. RNA for c-Jun was detected at all ages in both normal and transgenic mice (Fig. 7.4). In the ND4 mice, a sharp increase in expression occurred at 1 month of age but then decreased thereafter and remained at normal levels through 8 months of age (Fig. 7.4). RNA for c-Fos was also detected in both normal and transgenic mice (Fig. 7.5). The amount of RNA in transgenic animals was comparable to normal mice from 5 days to 2 months of age. After 3 months of age, these levels gradually increased and reached a plateau at approximately 2 fold higher than normal (Fig. 7.5). Both c-Jun and c-Fos expression were increased in the ND4 mice which suggests that these proteins may be involved in upregulating MMP expression in the ND4 mice. Although c-Fos expression seemed to correlate best with the onset of disease, the temporary increase in c-Jun at 1 month of age may be involved in early processes of the disease. Since AP-1 can also be activated by c-Jun homodimers, the increase in c-Jun at 1 month of age may activate MMPs as an early response to disease in the ND4 mice.

Developmental expression of TIMP-1 in normal and transgenic mouse brain

Since TIMPs naturally regulate the activity of MMPs, the expression of TIMP-1 was examined in normal and transgenic mice. TIMP-1 RNA was detected in both normal and transgenic mice. The amounts of RNA were the same between normal and ND4 mice and remained constant with age (Fig. 7.6, A and B). Thus, the expression of TIMP-1 was not increased over normal levels in transgenic mouse brain.
Figure 7.4: Developmental expression of c-Jun RNA in mouse brain. Ratio of c-Jun RNA in transgenic mice relative to normal mice. (*error bars = standard deviation from 3 blots*)

Figure 7.5: Developmental expression of c-Fos RNA in mouse brain. Ratio of c-Fos RNA in transgenic mice relative to normal mice. (*error bars = standard deviation from 3 blots*)
Figure 7.6: Developmental expression of TIMP-1 RNA in normal and transgenic mouse brain. A) normal; B) transgenic.
Overexpression of TIMP-1 reduces the clinical severity of disease in the ND4 transgenic mice

Since MMP expression was increased in the ND4 mice, we postulated that inhibition of MMP activity by overexpressing TIMP-1 in the brain will result in a less severe phenotype in demyelinating animals. To demonstrate this, transgenic mice overexpressing TIMP-1 in brain (Kruger et al., 1998) were crossed with ND4 transgenic mice. Since the TIMP-1 transgenic mice (C57 black line) and the ND4 transgenic mice (CD1 line) were of a different background, the offspring from this mating would create a genetically different mouse line. Consequently, the double transgenic mice (TIMP-1+DM20+) were compared to both ND4 mice and the litter mates which overexpressed DM20 (DM20 overexpressors). These animals were clinically scored as described in the Methods and the progression of disease was plotted in figure 7.7. Preliminary results showed that the clinical progression of DM20 overexpressing mice increased gradually from 3 months to 5 1/2 months of age (Fig. 7.7B). This increase in scores was neither as rapid nor as high as that of the ND4 mice perhaps the result of different genetic backgrounds (Fig. 7.7A). The double transgenic mice (TIMP-1+DM20+) were resolved into two populations in approximately a 50:50 ratio. One population of double transgenic mice (Fig. 7.7C) with the same clinical progression of its DM20 overexpressing litter mates showed no effect whereas a second population (Fig. 7.7D) showed a much less severe phenotype. These findings have since been confirmed in the laboratory using another set of (TIMP-1 and ND4) double transgenic mice. It has been postulated that the two populations of double transgenic mice may be due to different gene dosages or to gene silencing (Garrick et al., 1998). This is currently being investigated in the laboratory. The second population indicated that inhibition of MMP activity can improve the clinical phenotype.
**Figure 7.7** : Clinical progression of TIMP-1/DM20 double transgenic mice. Transgenic mice overexpressing TIMP-1 were mated with ND4 transgenic mice to produce TIMP-1+DM20+ double transgenic mice. The clinical progression was scored every second day and the sum of scores per week were plotted against age. DM20 overexpressing litter mates were also scored and compared with the double transgenic mice. Two populations of TIMP-1+DM20 double transgenic mice were found. A) Δ ND4 transgenic mice; B) DM20 overexpressors (~7 mice); C) TIMP-1+DM20+ transgenic mice (Pop. 1; ~7 mice); D) TIMP-1+ DM20+ transgenic mice (Pop. 2; ~7 mice). (error bars = standard deviation).

**Effect of paclitaxel on the expression of stromelysin-1 RNA in transgenic mice**

To determine if paclitaxel can affect the RNA expression of MMPs in the ND4 mice, stromelysin RNA levels were measured in the brains from treated transgenic animals (refer to Methods). RNA was extracted from the brains of paclitaxel treated transgenic...
mice immediately after the last injection at 4 months of age and two months after the last injection at 6 months of age. The levels of stromelysin-1 RNA present in these samples were measured by Northern blot as described in the Methods. Compared with untreated transgenic mice at 4 months of age, stromelysin-1 RNA levels were unchanged in all three paclitaxel treated transgenic mice (Fig. 7.8). In paclitaxel treated ND4 mice at 6 months of age, the RNA levels in two animals were slightly elevated over levels in the transgenic control while in a third mouse, the amount was slightly decreased (Fig. 7.8). In spite of these differences, no major reduction was observed either immediately after the last injection of paclitaxel or two months afterwards. Therefore, the high levels of stromelysin-1 RNA in transgenic mice were unaltered by treatment with paclitaxel. However, when expressed as a ratio relative to the normal control of the same age, the paclitaxel treated transgenics at 6 months of age were 2-3 times greater than at 4 months of age (Table 7.2). This relative increase of stromelysin expression may reflect the need for remodelling during remyelination in the 6 month old transgenic mice treated with paclitaxel.

Table 7.2: Relative ratio of stromelysin RNA in Paclitaxel treated and untreated ND4 transgenic mice. Expression levels of stromelysin RNA in transgenic mice treated and untreated with Paclitaxel at 4 and 6 months of age were normalized to levels in age matched normal mice and reported as relative ratios.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative ratio of RNA *(4 months)</th>
<th>Relative ratio of RNA *(6 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic (untreated)</td>
<td>7.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Treated Transgenic (A)</td>
<td>7.0</td>
<td>12.8</td>
</tr>
<tr>
<td>Treated Transgenic (B)</td>
<td>9.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Treated Transgenic (C)</td>
<td>9.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

* Ratio = (transgenic/normal)
Figure 7.8: Stromelysin RNA expression levels in mouse brains.

A, B, and C are samples from transgenic mice treated with Paclitaxel. Samples were taken immediately after the last injection (4 months of age) or two months after the last injection (6 months of age). Normal and transgenic age matched control mice were not treated with the drug (N and T respectively). All values were normalized to GAPDH RNA levels in the respective lanes. (*error bars = standard deviation*).

Discussion

In this chapter, the expression of MMPs in the ND4 transgenic mouse was examined to determine if these enzymes were involved in the disease process. Using Northern blot to detect MMP transcripts, an increase in stromelysin-1 (MMP-3) expression in the brain from ND4 mice was found to correlate with the onset of disease. This increase could be due to upregulation of MMP transcription by the transcription factor AP-1 which consists of c-Jun and c-Fos proteins. This was supported by the increase in c-Fos RNA.
which correlated with the onset of signs and by the increase in c-Jun RNA levels at 1 month of age. Expression of the natural inhibitor of MMPs, TIMPs (TIMP-1), was also measured to determine if TIMP-1 RNA was increased in response to the increase in stromelysin RNA levels. Normal levels of TIMP-1 RNA in the ND4 mice suggested TIMP-1 RNA was not upregulated during the course of disease in the ND4 mice. Whether an overexpression of TIMP-1 in the brains of ND4 mice could reduce clinical severity was addressed by mating transgenic mice overexpressing TIMP-1 in brain with ND4 mice. A population of double transgenic mice with reduced clinical signs indicated that inhibition of MMP by TIMP-1 can attenuate the clinical signs. The role played by MMPs in demyelinating disease was alternatively examined by treating ND4 mice with paclitaxel. Since paclitaxel has been shown to reduce AP-1 dependent expression of MMPs in cultured chondrocytes, it was postulated that this drug may reduce stromelysin-1 expression in the ND4 transgenic mice. The level of stromelysin-1 RNA was not reduced upon treatment of ND4 mice with paclitaxel. In fact the relative amounts were increased which would be expected if remyelination was taking place at 6 months of age.

The findings presented here implicate stromelysin-1 upregulation in the process of disease in the ND4 mice. The increase in stromelysin-1 expression may be involved in the activation cascade of other MMPs. Whether this MMP plays a negative or a positive role in demyelination remains to be determined. Based on the actions of MMPs in the degradation of components of the BBB (Woessner, 1994; Murphy, 1995) and of MBP into encephalitogenic fragments (Gijbels et al., 1993; Chandler et al., 1995), the natural conclusion would be that MMPs have a role in promoting demyelinating disease. However, in a recent study, an increase in MMP activity was shown to occur during oligodendrocyte process extension, changes in cell morphology, and remodeling (Uhm et al., 1998). It is possible that the increased levels of stromelysin-1 found in ND4 mice even with paclitaxel treatment may be required when recovery from myelin degradation begins
with remyelination. Thus MMPs appear to have functions in both demyelinating and remyelinating processes, which can occur at the same time in demyelinating disease (Prineas and Connell, 1979; Prineas et al., 1993). Although we have implicated stromelysin expression in the process of disease, the increase in expression found in ND4 mice may be due to degradative processes induced by proliferating astrocytes, invading macrophages, and lymphocytes as well as attempts at remyelination.
Chapter 8. Remyelination in Paclitaxel Treated ND4 Transgenic Mice

Introduction

Remyelination in the demyelinating disease Multiple Sclerosis (MS) was first suggested as early as 1965 by Perier and Gregoire (Perier and Gregoire, 1965). In their study which was later supported by Suzuki et. al. (Suzuki et al., 1969), thinly myelinated axons which they attributed to remyelination were observed at the edge of the demyelinated plaques using electron microscopy (EM) (Perier and Gregoire, 1965). However, remyelination in the central nervous system was not widely accepted until the late 1970's. In a study of the thinly myelinated axons in chronic plaques from MS patients, Prineas and Connell (Prineas and Connell, 1979) found that two types of internodes were present. The first type was not uniform in thickness along its length while the second type was uniform along its length but shorter (Prineas and Connell, 1979). They concluded that the latter type of internode was due to remyelination by oligodendrocytes. Since then, remyelination has been morphologically characterized by axons with thin myelin sheaths of uniform thickness in length. Occurring at the edge of plaques, these internodes are short in length and often form heminodes instead of paranodes at its ends (Prineas and Connell, 1979). Although remyelination in the CNS is now well accepted, the origin of myelinating cells involved in remyelination has become the centre of debate.

Several different sources of oligodendrocytes for remyelination have been suggested (Raine, 1997). The first source is thought to be mature non-mitotic oligodendrocytes (Raine, 1997). The second is mature cells that de-differentiate, divide, and repopulate demyelinated regions (Raine, 1997). De-differentiation of oligodendrocytes may be stimulated by C5b-9, a component of the complement system. This component has been shown to activate expression of the immediate early genes \textit{c-jun} and \textit{c-fos} and also induce oligodendrocytes to enter into the S phase from the resting G1/G0 phase \textit{in vitro}.
(Rus et al., 1996; Rus et al., 1997). However, the assumption made in these two theories is that oligodendrocytes survive a demyelinating attack and are thereby able to re-form myelin within lesions. The survival of oligodendrocytes remains a controversial point since this has only been shown in induced animal models of demyelinating disease (Herndon et al., 1977; Ludwin, 1978; Ludwin, 1979; Arnella and Herndon, 1984) and in some studies on demyelinated plaques from patients (Raine et al., 1981; Ghatak et al., 1989). In one of these studies, Raine et. al. examined a brain biopsy sample from a patient suffering from chronic MS by light and electron microscopy and found an abundance of oligodendrocytes within the lesion (Raine et al., 1981). These authors concluded that myelin alone was the target in MS as suggested in our mechanism (Fig. 6.9). However, oligodendrocytes were identified morphologically which is normally difficult to define and discern from leukocytes by this method. Alternatively, Prineas et. al. used antibodies for oligodendrocyte markers to detect these cells by immunohistochemistry (Prineas et al., 1989; Prineas et al., 1993). Contrary to the study by Raines et. al., they found that oligodendrocytes were reduced or absent in MS lesions (Prineas et al., 1993). Prineas et. al. suggested that both myelin and oligodendrocytes were targeted in demyelinating disease. The survival of oligodendrocytes in MS lesion remains to be resolved.

De-differentiation and proliferation of mature oligodendrocytes has been demonstrated using animal model systems. Upon examining brain tissue mechanically destroyed in normal mice, Ludwin and Bakker found that a small population of oligodendrocytes were capable of $^3$H-thymidine uptake (Ludwin and Bakker, 1988). This indicated that mature oligodendrocyte were capable of proliferating in response to injury in a normal system. Oligodendrocytes were also shown to proliferate after induced demyelination in animal models (Herndon et al., 1977; Ludwin, 1978; Ludwin, 1979; Arnella and Herndon, 1984). However, direct evidence for oligodendrocyte mitosis and de-differentiation in MS tissue is difficult to obtain since most studies employ autopsy
material. Thus cell proliferation remains to be demonstrated for oligodendrocytes in MS lesions.

The presence of progenitor cells that repopulate demyelinated lesions, proliferate and remyelinate the lesions is a third possibility for a source of cells. This source of oligodendrocytes that participate in remyelination may arise from neural stem cells found in the brain which have the capacity to generate neurons, astrocytes and oligodendrocytes in vitro (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). Isolated from the ependymal cell layer lining the lateral ventricle, these cells give rise to immature neurons that migrate to the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Morshead et al., 1994). In response to spinal cord injury, these cells also generate astrocytes which participate in scar formation (Johansson et al., 1999). Although oligodendrocyte precursors have not yet been isolated in vivo, stem cells from the ependymal layer are a likely source of precursor cells for remyelination. There is evidence which suggests the presence of an oligodendrocyte precursor cell in MS lesions (Prineas et al., 1989). In fresh lesions, cells expressing oligodendrocyte markers were not stained for MBP or MAG, markers for a more developed oligodendrocyte. In older lesions, both MBP and MAG were found in addition to oligodendrocyte markers in cells within remyelinating regions (Prineas et al., 1989). An increase in the number of oligodendrocytes was also observed in these regions (Prineas et al., 1993). Findings from a study performed by Capello et. al. demonstrated that cells in remyelinating regions in MS plaques were developmentally less mature (Capello et al., 1997). Using in situ hybridization and immunohistochemistry they found that oligodendrocytes in remyelinating regions contained higher amounts of exon 2 containing MBP mRNAs and proteins. Products containing sequences encoded by exon 2 of the MBP gene (21.5 and the 20.2 kDa isoforms in human) are preferentially expressed in early development. Thus the high amounts of the mRNA or protein containing MBP exon 2 was indicative of a developmentally immature oligodendrocyte. Capello et. al.
concluded that the cells in remyelinating MS plaques were at an earlier stage of development (Capello et al., 1997) and suggested that an early myelinating system was recapitulated in the process of remyelination.

Although an early myelinating system is expected for remyelination, it may also be the reason why widespread remyelination of lesions is inhibited. This inhibition may be mediated by players involved in demyelination such as the macrophages and astrocytes. Within plaques, macrophages occur in abundance at the center of the lesion while astrocytes are typically found at the edges (Prineas et al., 1993). Macrophages in newly formed lesions are often observed under EM to contain myelin fragments, which stain positively for MBP by immunocytochemistry, and oligodendrocyte cell bodies (Prineas and Connell, 1978a; Prineas and Wright, 1978b; Raine et al., 1981; Prineas et al., 1993). Several studies have also shown activated astrocytes engulfing oligodendrocytes and myelin (Prineas et al., 1990; Ghatak, 1992). Using immunohistochemistry, Prineas et al. found that cells within astrocytes in MS lesions reacted to antibodies for the oligodendrocyte determinants 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) and the human natural killer cell epitope (HNK-1) but not MBP or MAG (Prineas et al., 1990). These authors suggested that the engulfed cells were non-myelinating, undifferentiated oligodendrocytes and proposed that the ingestion of premyelinating oligodendrocytes by astrocytes prevented successful remyelination in the plaques. Another means by which astrocytes inhibit remyelination is through the extension of processes from these cells which surround demyelinated axons (Ludwin, 1995). These processes form a physical barrier around axons thereby preventing remyelinating cells access to the axon. If the inhibitory effect of astrocytes is blocked, perhaps remyelination will be allowed to proceed.

Ideally, tissue from MS patients would be best to study the process of remyelination. Since most studies using MS tissue are performed on autopsy material, the
types of experimental procedures are limited. Alternatively, animal models induced to
demyelinate have been employed to study oligodendrocyte proliferation during
remyelination. However, demyelinating disease does not persist in these model systems
and the pathology of disease such as the chemically induced models is artificial compared to
MS. The effects of the inducing agent on oligodendrocytes could be very different
compared to MS. As a result, oligodendrocytes may be spared in these systems and
remyelination in these models may be different compared with MS. In contrast,
demyelinating disease in the ND4 transgenic mouse model is spontaneous, progressive,
and persistent, similar to MS. Biochemical changes in MBP found in MS have been
shown to occur in ND4 mice but not in EAE mice (Mastronardi et al., 1996a; Mastronardi
et al., 1996b). Thus this animal model can provide better insight into the process of
remyelination in MS.

In chapter 6, treatment of ND4 transgenic mice was shown to attenuate the
progression of clinical signs and to inhibit proliferating astrocytes. This inhibitory effect
on proliferating astrocytes by paclitaxel could increase the extent of remyelination in
demyelinating mice. This may explain the improvement in clinical disease. In this chapter,
the degree of remyelination was examined in ND4 transgenic mice treated with paclitaxel to
determine whether the inhibition of astrocytes in these animals promoted recovery.

Results

Normal and ND4 transgenic mice were treated with paclitaxel and sacrificed at 6
months of age as described in the Methods. For light microscopy studies on brain and
spinal cord sections, each animal was anesthetized and perfused through the left ventricle
with fixative. The fixed brain and spinal cord tissue were removed, post-fixed and washed
for sectioning as described. The samples were stained with Luxol-fast blue and examined
under light microscopy. For electron microscopy studies, mice were sacrificed by cervical
dislocation. The optic nerve was removed and fixed as described in the Methods. Sections and photographs were prepared by either C. A. Ackerley (Hospital for Sick Children, Dept. of Pathology, Toronto) or by Dr. S. Ludwin (Queen's University, Dept. of Pathology, Kingston).

**Ultrastructure of mouse brain and spinal cord sections**

Sections of the posterior column of the spinal cord and the superior cerebellar peduncle of the brain from normal mice, paclitaxel treated ND4 mice, and untreated ND4 mice at 6 months of age were prepared and stained to detect myelin. The posterior column from spinal cord of a normal 6 month old mouse is shown in Figure 8.1a. In this section, both small and large caliber axons were surrounded with thick, compact myelin (Fig. 8.1a, arrow). The myelinated fibers were densely arranged with few scattered astrocytes. The number and density of myelinated fibers was similar in sections of the posterior column from 6 month transgenic mice (Fig. 8.1b). However, the membrane around some axons was unusually thick indicating swelling of the myelin membranes. This suggested that the compaction and stability of myelin was disrupted. Although both small and large caliber myelinated axons were observed, loosely compact myelin and nude axons were also present. Astrocytes were more abundant in these sections compared with the normal sample. These findings were characteristic of ongoing demyelination in untreated ND4 mice. When sections of the posterior column from ND4 transgenic mice treated with paclitaxel were examined, astrocytes were also observed (Fig. 8.1c) but the amount of myelin staining in these sections was visibly different compared with samples from both normal and untreated transgenic mice (Fig. 8.1). The types of myelinated fibers were more variable with almost equal numbers of both thick and thinly myelinated axons (Fig. 8.1 c). This variability was due to the increase in thinly myelinated axons which suggests that remyelination was underway. Furthermore, myelin did not appear to be swollen as was
Figure 8.1 Light microscopy of the posterior column from mouse spinal cord. Sections of the spinal cord were taken from 6 month old mice, fixed, and stained with Luxol-fast blue. The posterior column was examined in sections from normal mice (a), ND4 transgenic mice (b) and ND4 transgenic mice treated with paclitaxel (c). Ast, astrocyte; A, myelinated axon; N, nude axon; S, axon with swollen myelin sheath; *, thinly myelinated axon; Arrows, compact myelin around axon. Bar =2μm, EM by Dr. S. Ludwin, Queen’s University, Dept. of Pathology, Kingston)
observed in the untreated ND4 mice (Fig. 8.1b, c). Thus myelin stability and compaction did not appear to be disrupted at this stage. Therefore, treatment of ND4 mice with paclitaxel appeared to promote remyelination in the posterior column of the spinal cord. To determine if the same effect could be found in another part of the CNS, nerve fiber tracts in the superior cerebellar peduncle were also examined.

Gathered in bundles of nerve fiber tracts, myelinated axons were more distinctive in sections of the superior cerebellar peduncle. This was observed in a section from this area of the brain from a 6 month old normal mouse (Fig. 8.2a). Axons were well arranged and well defined within the tracts each containing thick, compact myelin sheaths. Sections of the same area of the brain from 6 month old ND4 transgenic mice (Fig. 8.2b) did not contain the distinctive tracts observed for the normal samples. In contrast, very little compact myelin and an abundance of nude axons were detected. Myelinated axons were visibly reduced and randomly distributed throughout this sample clearly indicating demyelination (Fig. 8.2b). Upon treatment with paclitaxel, this effect seemed to be reversed as observed in sections of the superior cerebellar peduncle from 6 month old ND4 mice treated with paclitaxel (Fig. 8.2c). In these sections, the number of myelinated axons was increased with a reduction in the number of nude axons. Myelinated axons were clearly visible and appeared in organized bundles (Fig. 8.2a, c) but not as distinctive as in normal mouse samples (Fig. 8.2b, c). As observed in the spinal cord section from paclitaxel treated ND4 mice, both thick and thinly myelinated axons were detected within the nerve fiber tracts in the superior cerebellar peduncle. Although some swollen myelin was seen, the increase in thinly myelinated axons and the reduction in nude axons suggested remyelination was occurring in this animal. Thus the increase in the relative amount of compact myelin and thinly myelinated axons found in the different areas of the CNS in paclitaxel treated animals suggests that remyelination was increased in paclitaxel treated transgenic mice.
Figure 8.2  Light microscopy of the superior cerebellar peduncle from mouse brain. Section of the superior cerebellar peduncle from the brain of 6 month old mice were stained with Luxol-fast blue. Myelinated axons were arranged in nerve fiber tracts which appeared as bundles in sections from normal mice (a). These bundles were less visible in sections from ND4 transgenic mice (b). The bundles in ND4 transgenic mice treated with paclitaxel were more visible and contained both thick and thinly myelinated axons (c) indicating recovery and remyelination.

Ast, astrocyte; A, myelinated axon; N, nude axon; *, thinly myelinated axon. (Bar=3μm, EM by Dr. S. Ludwin, Queen’s University, Dept. of Pathology, Kingston)
Ultrastructure in optic nerve sections from paclitaxel treated and untreated mice

The structure and organization of myelin in normal and ND4 mice treated or untreated with paclitaxel was alternatively examine using EM. Optic nerve from 6 month old paclitaxel treated normal mice is shown in Figure 8.3a. Thick and well compact myelin was observed indicating that paclitaxel did not have deleterious effects on the myelin sheath. Both myelinated and denuded axons were observed in optic nerve sections from 6 month old untreated transgenic mice (Fig. 8.3b) and paclitaxel treated transgenic mice (Fig. 8.3c). In sections from untreated ND4 mice, the number of nude axons out numbered myelinated axons indicative of extensive demyelination (Fig. 8.3b). In contrast, the myelinated axons were more abundant in the section from paclitaxel treated ND4 mice (Fig. 8.3c). Visibly fewer nude axons were observed in these sections. With the presence of thinly myelinated axons among myelinated axons, the increase in myelinated axons, and fewer nude axon, remyelination appeared to be promoted and underway in treated mice.

EM sections prepared by Dr. Sam Ludwin et. al. (Queen's University, Dept. of Pathlogy, Kingston, Ontario) from another set of animals showed similar features. In this experiment, white matter in the corpus callosum from normal mice showed numerous axons with myelin of appropriate thickness (Fig. 8.4a). Oligodendrocytes in these sections appeared normal with oval nuclei containing smooth nuclear membranes (Fig. 8.4a) (Dr. S. Ludwin, Queen's University, Dept. of Pathlogy, Kingston, personal communication). Irregular myelin profiles and an abundance of nude axons were observed in sections from untreated transgenic mice (Fig. 8.4b) indicating continual myelin breakdown. Thinly myelinated axons were also found in these sections evidence of attempted remyelination (not shown). The oligodendrocytes in these sections were mildly affected with no indication of cell death (Dr. S. Ludwin, Queen's University, Dept. of Pathology, Kingston, personal communication). In these cells, the nuclear membrane appeared notched with
Figure 8.3  Electron micrograph of optic nerve sections from mice. Optic nerves were removed from 6 month old mice, fixed, sectioned, and examined by electron microscopy. Samples examined were from normal mice (a), ND4 transgenic mice (b) and ND4 transgenic mice treated with paclitaxel (c). Ast, astrocyte; A, myelinated axon; N, nude axon; *, thinly myelinated axon. (Bar=1µm, EM by C. A. Ackerley, Hospital for Sick Children, Dept. of Pathology, Toronto)
Figure 8.4: Electron micrographs of axonal cross sections from: a) Corpus callosum from normal mouse; b) Corpus callosum from untreated ND4 transgenic mouse; c) Optic nerve from paclitaxel treated ND4 transgenic mouse. A, axon with myelin of normal thickness; N, nude axons; OL, oligodendrocyte; Arrows, thinly myelinated axons. (Bar = 1 μm. EM by Dr. S. Ludwin, Queen's University, Dept. of Pathology, Kingston)
numerous indentations while the cytoplasm contained dense bodies (Fig. 8.4b). Sections from Paclitaxel treated animals showed an increase in myelinated fibres and a decrease in nude axons. In these areas, many of the axons contained thin myelin sheaths providing evidence of remyelination (Fig. 8.4c). Although the oligodendrocytes in these sections had a notched nuclear appearance, this was not as severe as in untreated transgenic samples (Fig. 8.4c) (Dr. S. Ludwin, Queen's University, Dept. of Pathology, Kingston, personal communication). In comparison, the extent of myelin breakdown was more obvious and extensive in untreated transgenic mice. Thus the results from both EM experiments were in agreement with those observed by light microscopy on spinal cord and superior cerebellar peduncle.

Summary

Since proliferating astrocytes are thought to play an inhibitory role in remyelination by blocking oligodendrocytes or precursor cells from forming myelin on denuded axons, it was postulated that by targeting proliferating astrocytes in demyelinating disease, the progress of recovery may be enhanced. This proposal was supported by the reduction in the number of astrocytes and the attenuation of clinical signs in ND4 mice treated with paclitaxel (Chapter 6). In this chapter, several methods were used to examine the effects of paclitaxel treatment on remyelination. Light microscopy sections stained for myelin demonstrated an increase in myelinated axons and fewer nude axons in 6 month old paclitaxel treated transgenic mice. This was observed in both the spinal cord and in the superior cerebellar peduncle of the brain. The same ultrastructure was found in the optic nerves and brain sections using electron microscopy. Although the density of myelinated axons was not as high as in normal mice, myelinated fibers in sections from treated ND4 mice were more visible than in untreated transgenic mice. The thickness of myelin sheaths in these sections were not as uniform compared with normal samples. Both thick and
thinly myelinated axons were found next to nude axons. Since thinly myelinated axons accompanied by fewer nude axons were more abundant in treated ND4 mice than in untreated transgenic animals, this finding suggested that paclitaxel promoted remyelination. Experiments are currently underway to confirm that remyelination is occurring in treated ND4 mice by examining the expression levels of MBP isoforms with sequences encoded by exons 2 of the MBP gene (21.5 and 17 kDa). An increase in the expression of both RNA and protein containing a sequence encoded by exon 2 has been shown to be associated with remyelinating lesion in MS tissue (Capello et al., 1997). Therefore, if remyelination is occurring in treated transgenic mice, an increase in RNA and protein containing an exon 2 encoded sequence is expected. Since ND4 treated mice showed partial recovery by 6 months of age, other experiments are underway to determine if paclitaxel treated ND4 mice can survive and fully remyelinate if maintained for 10-12 months of age. From the result obtained so far, paclitaxel appears to enhance remyelination in the ND4 transgenic mice. This drug may function by inhibiting processes that prevents remyelination such as the role played by proliferating astrocytes.
Chapter 9. Discussion

The approach towards understanding the mechanism of demyelination in MS has traditionally been from an autoimmune perspective because of the players that have been found to be involved in the disease process such as the T-cells, B-cells, proliferating astrocytes, and macrophages. These cells engulf or respond to myelin, oligodendrocytes, or epitopes from myelin and initiate a cascade of signals resulting in the activation of the immune system. This is enhanced by the secretion of factors such as cytokines (deVries et al., 1996; Merrill and Benveniste, 1996; Navikas and Link, 1996; Giraudon et al., 1997; deBoer and Breimer, 1998) and MMPs (Rosenburg et al., 1992; Leppert et al., 1995; Rosenberg et al., 1995; Leppert et al., 1996; Stuve et al., 1996; Clement et al., 1997) which activate and allow the infiltration of lymphocytes and macrophages into the CNS through the BBB. However, our knowledge of this mechanism and this approach to the disease does not address how demyelinating disease is initiated. Although many researchers will argue that increased immune susceptibility may be responsible for disease in some patients, other scientists believe that demyelination arises from an inherent defect within the oligodendrocyte which disrupts the proper formation of myelin (Rodriguez et al., 1993; Moscarello et al., 1994). Whether this defect is genetically linked or the result of environmental influences remains to be resolved but recent studies on the charge microheterogeneity of myelin basic protein in CNS myelin from MS patients has implicated the loss of positive charges on MBP in the destabilization of myelin through the accumulation of citrullinated MBP (MBP-Cit6 or C8) (Moscarello et al., 1994). This change in MBP was not found in animals with EAE (Mastronardi et al., 1996a), a model for the immunological response in demyelinating disease, but was found in the ND4 transgenic mice, a spontaneously demyelinating animal model (Mastronardi et al., 1996b). This suggests that the change in MBP is not a consequence of autoimmune attack and that it
precedes an immune response. Since increased MBP-Cit6 was also found in ND4 mice and since the development of disease in these animals occur as they age, the ND4 transgenic mice represent an excellent model in which to study the progression and development of demyelinating disease. In this thesis, we endeavored to elucidate the reactions leading to myelin destabilization in the ND4 transgenic mice. The results from our study have provided us with information from which we propose a mechanism of demyelination based on changes in the post-translational modification of myelin proteins. These changes could have negative effects on myelin integrity and stability.

Post-translational modifications are powerful methods by which proteins can be regulated. Some modifications control enzymatic activity, for example, RNase activity requires the formation of disulfide bonds (Cuatrecasas et al., 1968; Anfinsen, 1973), phosphorylation of ser 16 in glycogen phosphorylase b is slowed upon deimination of arg 16 which affect its affinity for AMP binding and the formation of tetramers (Luo et al., 1995; Eronina et al., 1996), and kinase activity in signal transduction pathways is dependent on cycling of phosphorylation states. Other modifications control protein localization and orientation, for example, palmitoylation of G-proteins aid in their localization to the cell membrane (Mumby, 1997), while palmitoylation of the β-adrenergic receptor maintains its conformation permitting the coupling of the receptor with G-proteins (O'Dowd et al., 1989). Post-translational modifications of proteins can also lead to disease. Several recent studies have implicated deiminated arginyl residues in filaggrin as the epitope involved in the autoimmune disease rheumatoid arthritis (Schellekens et al., 1998; Girbal-Neuhauser et al., 1999). In light of the changes found in MBP charge isomers from MS patients (Moscarello et al., 1994) and from ND4 transgenic mice (Mastronardi et al., 1996b) and in PLP and DM20 from these animals as presented in this thesis, demyelination may be considered to be a "post-translational modification disease".
From the changes found in the post-translational modification of myelin proteins, we propose a mechanism of demyelination which is multi-faceted involving two major parts. The first part deals with changes within the oligodendrocyte and myelin resulting in myelin instability while the second part encompasses the response to these changes by extracellular components and other cells. The second part was derived largely from findings currently in the literature while the first part was based on the findings presented in chapters 3 through 5 which focused on the changes in PLP and DM20 protein levels and their post-translational modifications. Summarized in Figure 9.1, these findings resulted from our initial attempt to ascertain the reason behind the reduction in PLP and the increase in DM20 protein observed earlier in ND4 mice (Mastronardi, 1996; Barrese et al., 1998).

Since the ND4 mice contained 70 copies of a normal DM20 cDNA transgene, each under the transcriptional control of a normal PLP promoter, our initial approach was to examine these mice at the level of transcription factor binding. Analysis of the transcriptional complexes formed with oligonucleotide probes corresponding to transcriptionally active sequences found in the PLP promoter demonstrated that the transgenes did not compete with the PLP gene for binding of regulatory proteins (Figs. 3.4 and 3.8). Located in the proximal region of the PLP promoter, a region sufficient for maximal transcriptional activity of the PLP gene (Berndt et al., 1992; Cambi and Kamholz, 1994), the probe sequences used were two of five that are transcriptionally active (Berndt et al., 1992). Of particular importance was the oligo 4 sequence, which is a binding site for a myelin specific transcription factor (MyTi-1) (Kim and Hudson, 1992). Since the factors which bind to oligo 4 have been shown to be required for the specific transcription of PLP (Kim and Hudson, 1992) and since the transgene contains a normal PLP promoter, these factors are also expected to participate in the specific transcription of the transgene. Normal levels of the complexes formed using oligo 4 in transgenic samples (Fig. 3.8) suggested that the transgenes did not sequester transcription complexes specific to the PLP gene.
This conclusion was supported by the amount of endogenous DM20 and PLP mRNAs measured in ND4 mice which were the same as those in normal mice.

*Figure 9.1: Summary of the changes found in MBP, PLP and DM20 in ND4 transgenic mice. ( * -Hyper fatty acylated)*

In ND4 transgenic mice, the levels of PLP and endogenous DM20 mRNA were unchanged throughout development in spite of the high amounts of transgene DM20 mRNA. The 3 fold increase in DM20 protein corresponded with the 4 fold increase in transgene DM20 mRNA indicating that the majority of the DM20 protein was derived from the transgene. On the other hand, PLP mRNA levels did not reflect the decrease in protein and could not be explained by a decrease in stability since the half-life of mRNA from the PLP gene was also unaffected (Table 4.1). Normal expression levels and rate of decay for mRNA transcribed from the PLP gene suggested that the rate of transcription was also
unaffected in the ND4 mice although this will need to be confirmed by transcription rate experiments. Therefore the changes in PLP and DM20 protein levels in the ND4 mice appear to be the result of a post-transcriptional event(s).

Changes in post-translational modification were found in PLP and DM20 isolated from the brains of ND4 mice. The finding that both DM20 and PLP from 1 month old ND4 transgenic mice were hyperacylated may explain the low amounts of DM20 observed in myelin at 1 month of age (Barrese et al., 1998) as well as the low amounts of PLP measured in both whole brain homogenate and myelin (Mastronardi, 1996; Barrese et al., 1998). Since covalently bound palmitoyl groups have been shown to target proteins such as G-proteins and Rhodopsin to membranes and to anchor and aid their interactions with lipids and other proteins (Milligan et al., 1995; Morello and Bouvier, 1996), it is speculated that the covalently bound fatty acids on PLP and DM20 serve the same function by maintaining an optimum orientation and interactions in myelin. This may also affect the capacity of PLP to act as an ion channel and result in changes in ion distribution within myelin. In the ND4 transgenic mice, an increase in covalently bound fatty acids could alter the targeting of PLP or DM20 to myelin diverting the proteins to lysosomes for degradation. As a result, the amount of PLP in whole brain homogenate would be low. Since DM20 protein levels are expectantly high in whole brain homogenate, the proportion of this protein degraded may not be as easily detectable as with PLP. However, when DM20 levels are lower, for example, at 1 month of age, this reduction can be detected as observed by the absence of DM20 protein in myelin at this age. To resolve the problem of degradation, the subcellular organization of PLP and DM20 protein will need to be studied in oligodendrocytes from ND4 mice.

The re-distribution of PLP and DM20 protein away from myelin may be a partial effect since some PLP and DM20 was found in myelin (Barrese, 1996). Once assembled into myelin, the excess covalently bound fatty acids on PLP and DM20 may disrupt their
orientation and interactions within the lipid bilayer. Although the role(s) of PLP/DM20 in myelin remains to be defined fully, studies of PLP deficient mice have suggested that PLP/DM20 play a role in the maintenance of the structural spacings in myelin (Boison and Stoffel, 1994; Rosenbluth et al., 1996; Klugmann et al., 1997). Since PLP-bound fatty acids represent a proportion of fatty acyl chains in myelin equaling that of phosphatidylinositol (Bizzozero et al., 1991b), these covalently bound fatty acids may have a role in myelin organization. In a study by Bizzozero and Lees, PLP isolated from patients suffering from classical adrenoleukodystrophy (ALD) and chronic adrenomyeloneuropathy (AMN) was found to contain increased levels of bound very-long chain fatty acids (VLCFA) (Bizzozero et al., 1991b). These authors speculated that the alteration in fatty acid content contributed to the pathogenesis of these diseases, both of which are characterized by demyelination. With a similar perspective, results from a study by Bernheimer et. al. using brain tissue from ALD, MS and systemic lupus erythematosus patients suggested that an excess of VLCFA in myelin lipids can impair membrane stability and induce demyelination (Bernheimer et al., 1983). In the ND4 transgenic mice, a disruption of the normal structure and function of PLP/DM20 in myelin by the increase in covalently bound fatty acid per mole of protein may result in a loss of myelin stability (Fig. 9.2). As a result, myelin will be more susceptible to attack and disruption.

Previous studies on ND4 mice have revealed that the quantity of both MBP gene transcripts and total proteins were normal throughout development and that the only change in MBP was the loss of positive charges due to post-translational modification (Mastronardì et al., 1996b). In a less stable myelin, MBP could be a potential target for attack or further modification (Fig. 9.2). Disruption of the bilayer may liberate enzymes from constraints imposed by a compact myelin structure permitting better access of the enzyme to substrates. Increased exposure of MBP may facilitate modification by PAD which has been localized to both compact myelin and the periaxonal region of myelin (Pritzker, 1996). The activity of
Figure 9.2: Proposed mechanism of myelin destabilization in ND4 transgenic mice. Changes in lipid/protein interaction due to hyperacylation of PLP and DM20 alters the organization of lipid within myelin and the stability of the membrane. This reduces the compaction of myelin and increases its susceptibility to attack. A potential target for attack could be MBP. Destabilization of compact myelin could exposed MBP to modification by PAD and digestion by cathepsin D or MMPs.
this enzyme has been found to be increased in 3 month old ND4 mice (Mastronardi et al., 1996b) which may explain the elevated levels of MBP-Cit6 in myelin from ND4 mice. A more extensive study to determine the change in PAD activity over the course of development in ND4 mice is currently underway in our laboratory. The accumulation of MBP-Cit6 in myelin could reduce the electrostatic interactions that are thought to mediate MBP’s function in maintaining myelin compaction. As a result, increased MBP-Cit6 levels could further destabilize and loosen the multi-lamellar bilayers in myelin. This was supported by the observation of extensive myelin vacuolation in electron micrographs from ND4 mice (Mastronardi et al., 1993) and in MS tissue (Wood et al., 1975; Rodriguez and Scheithauser, 1994). With increased citrulline content, MBP may further disrupt the bilayer and promote vesiculation as suggested by the fragmentation of acidic lipid vesicles by MBP-Cit18 (Boggs et al., 1999). A correlation of the extent of deimination of MBP with disease severity was also demonstrated in homozygous ND3a transgenic mice. These mice have less citrullinated MBP and a more gradual onset of disease compared with ND4 transgenic mice (Mastronardi et al., 1996c). Thus an increase in MBP post-translational modification resulting in an increase in citrulline content could result in a less stable, less compact myelin that is more susceptible to breakdown.

With a loosely compacted myelin sheath, myelin epitopes become more accessible to digestion by enzymes such as cathepsin D or a 60 kDa MMP (Fig. 9.2). Both enzymes have been found in myelin and were shown to release encephalitogenic peptides upon digestion of MBP (Brostoff et al., 1974; Whitaker and Seyer, 1979; Azaryan et al., 1983; Chandry et al., 1989; Chandry and Glynn, 1990). Cao et al. has demonstrated that MBP-Cit6 isolated from white matter from MS patients was digested by cathepsin D 4 times faster than MBP-Cit0 or C1 while MBP-Cit18 was digested 35 times faster than MBP-Cit0 (Cao et al., 1999). The presence of citrulline in MBP may prove to be an important factor in the activation of the immune system since antibodies specific to citrulline containing
epitopes within filaggrin have been found in the "autoimmune disease" rheumatoid arthritis (Girbal-Neuhauser et al., 1999). Therefore, the increase in MBP-Cit6 may propagate disease by enhancing the amount of encephalitogenic peptides generated, enhancing an immune response to myelin epitopes and destabilizing myelin compaction. Since MBP-Cit6 has been localized to the outer surface of the myelin bilayer (McLaurin et al., 1993) and has weaker electrostatic interactions with acidic lipids, it could be shed from loose myelin and taken up by cells such as astrocytes (Fig. 9.2). Thus the changes in the post-translational modification of MBP could stimulate responses to myelin and oligodendrocytes by astrocytes and cells from the immune system. These results suggest that demyelination in the ND4 mice may not be the direct effect of a single genetic lesion but rather the change in the relative levels and biochemistry of major myelin proteins.

Changes in the post-translational modification of PLP, DM20 and MBP have been proposed to lead to myelin instability and increased susceptibility to breakdown in the first part of the mechanism of demyelination. However, several questions remain to be resolved regarding the circumstances of the changes in PLP and DM20 protein. Although an explanation for low PLP levels may be the targeting of this protein for degradation, whether this occurs in the ND4 mice and whether it is directly due to the increase in covalently bound fatty acids on PLP remains to be shown. Also, why would PLP, the major protein in myelin, be targeted for degradation? Based in studies of animals with mutations in the PLP gene, it has been suggested by Knapp et al. that an abundance of DM20 mRNA or protein may be a signal for a developmentally immature cell (Knapp, 1996). In response to prolonged elevation of DM20 mRNA or protein, the oligodendrocyte may target PLP, which is characteristically abundant in mature myelin, to degradative pathways by hyperacylation in order to maintain a developmentally immature system. DM20 may also be targeted for degradation in attempt to control its abundance. An alternate explanation for low PLP levels could be translation competition between
DM20 mRNA and PLP mRNA. *In vitro* translation rate experiments could provide insight into this possibility. In this situation, one must determine whether translational competition and hyperacetylation of PLP and DM20 protein are independent processes. Though we have proposed a series of reactions occurring within oligodendrocytes and myelin that could lead to demyelination, the circumstances of the changes observed remains to be clarified for a better understanding of each reaction.

The second part of the mechanism illustrated in Figure 9.3 outlines the response of other cells and extracellular components to an abnormal myelin. MBP or MBP peptide released from myelin has been shown to be taken up and to activate cells such as astrocytes (Bologa et al., 1985; Owh et al., 1997), macrophages (Carbone et al., 1983; Williams et al., 1994) or lymphocytes (Nag et al., 1993). Using an astrocytoma cell line, Owh et al. demonstrated that these cells internalize MBP with a concomitant increase in the cell adhesion molecule CD44. They also showed that MBP binds directly to CD44 (Owh et al., 1997). Isoforms of this molecule expressed in astrocytes (DaCruz et al., 1993; Moretto et al., 1993), oligodendrocytes (Moretto et al., 1993) and lymphocytes (Stamper and Woodruff, 1976; Gallatin et al., 1983; Jalkanen et al., 1986) are believed to function as homing molecules (Jalkanen et al., 1986). Thus, the binding of MBP to CD44 may be the signal for astrocyte activation and homing of astrocytes to myelin. At the same time, upregulation of CD44 may also promote the interaction of activated astrocytes with lymphocytes. This interaction may facilitate the activation of the immune system since astrocytes are known to ingest myelin debris and express class II MHC molecules (Hickey and Kimura, 1988; Williams et al., 1993), the mechanism by which encephalitogenic epitope are believed to be presented to T-cells (Prineas and Graham, 1981; Fontana et al., 1984; Hohlfeld, 1989; Williams et al., 1993). Activation of lymphocytes, astrocytes and macrophages results in cytokine secretion which triggers responses that mediate injury to myelin such as the proliferation of other lymphocytes, the production of myelin specific
Figure 9.3: Potential mechanism of demyelination in the ND4 transgenic mice. The inability of oligodendrocytes to maintain a compact myelin results in the release of MBP or MBP peptides which can be taken up by astrocytes or can activate macrophages and lymphocytes. Uptake and internalization of MBP by astrocytes occurs through the cell adhesion molecule CD44. This stimulates astrogliosis, CD44 expression which may facilitate astrocyte homing to oligodendrocytes and astrocyte interaction with lymphocytes, and the expression of MBP presenting MHC class II molecules which induces an immune response in lymphocytes. In response lymphocytes secrete cytokines
which can stimulate synthesis of MMPs. MMPs enhance injury by further digesting MBP in myelin and components of the blood brain barrier allowing vascular lymphocytes and macrophages to infiltrate into the brain. Cytokines also stimulate the immune response by increasing proliferation and stimulating antibody production in B-cell. Influx of macrophages increases the uptake and engulfing of myelin and myelin debris. Components of the complement system also become involved in either myelin damage or at a later stage of disease, recovery.

antibodies (Jelinek and Lipsky, 1987; Lipsky, 1989; Mizel, 1989), and the synthesis and activation of MMPs (Ries and Petrides, 1995).

MMP are extracellular endoproteases that could contribute to myelin degradation and mediate the inflammatory response. Their expression and activity has been found to be increased in MS patients (Gijbels et al., 1992; Gijbels et al., 1993; Gijbels and Steinman, 1994a; Maeda and Sobel, 1996) and to correspond with the onset of clinical signs in both EAE mice (Gijbels et al., 1992) and ND4 animals (Chapter 7). These enzymes, produced by activated astrocytes and monocytes (Gijbels et al., 1994b; Cuzner et al., 1996), have been shown to produce encephalitogenic peptides upon digestion of MBP (Gijbels et al., 1993; Chandler et al., 1995) and to digest components forming the BBB. In vitro transmigration of T-cells has been shown by several groups to be MMP dependent (Rosenburg et al., 1992; Rosenberg et al., 1995; Leppert et al., 1996; Stuve et al., 1996; Clement et al., 1997). Thus MMPs may propagate injury to myelin by releasing MBP peptides and by mediating infiltration of lymphocytes, vascular macrophages, and components of the complement system.

The complement system comprises of a series of protein complexes that function in the recognition and destruction of pathogens and the handling of immune complexes (Morgan and Gasque, 1996). Through the recruitment of phagocytes to sites of
destruction, the complement complexes stimulate cell lysis and inflammation for clearance of pathogens. The complement system also assembles a group of membrane associated molecules (C5b-7, C5b-8 and C5b-9) collectively referred to as the terminal complement complexes (TCC) (Bhakdi and Tranum-Jensen, 1983). In the absence of cell lysis, insertion of these complexes into the cell membrane can induce intracellular signalling cascades and a variety of biological responses. Inappropriate activation of the complement system has been implicated in the pathology of numerous diseases including MS. However, the roles suggested for the complement system in MS have been conflicting especially in light of recent findings. Early evidence implicating the complement system in the pathogenesis of MS was suggested by the discovery of soluble complement activation complexes in CSF from MS patients and the deposition of lytic membrane attack complexes at the edges of active plaques (Mollnes et al., 1987; Compston et al., 1989; Yam et al., 1989). In EAE induced animals, demyelination was inhibited by depletion of serum complement using cobra venom factor (CVF) (Linnington et al., 1989) or the soluble complement receptor (sCR1) (Piddlesden et al., 1994). Furthermore, assembly of C5b-8 and C5b-9 on myelin resulted in hydrolysis of MBP through the activation of a Ca\(^{2+}\)-dependent neutral protease (Vanguri and Shin, 1988). In spite of these findings, the promotion of destructive processes in MS by the complement system has been challenged by recent discoveries implicating a role in recovery. In the absence of cell lysis, activation of the TCC component C5b-9 has been shown to stimulate degradation of MBP and PLP mRNA (Shirazi et al., 1993), markers of differentiated oligodendrocytes. At the same time, C5b-9 induced expression of the immediate early genes c-jun and c-fos through the mitogen activated protein kinase (MAP-Kinase) pathway (Shirazi et al., 1993; Rus et al., 1997). Activation of this signaling cascade by TCC induced oligodendrocytes to enter into the S phase of the cell cycle from a resting G1/G0 phase (Rus et al., 1996). Furthermore, complement activation increased expression of the RGC-32 gene (response gene to
complement -32) in oligodendrocytes, a gene that is postulated to have a role in cell cycle activation (Badea et al., 1998). Based on these recent findings, the complement system may stimulate remyelination in MS through the de-differentiation of mature oligodendrocytes into precursor cells. It is possible that in early stages of demyelination, the complement system enhances inflammatory responses and promotes myelin degradation but with time and clearance of myelin debris, the complement system stimulates remyelination by promoting de-differentiation of surviving or surrounding oligodendrocytes.

As discussed in chapter 8, remyelination is thought to arise from either the stimulation of de-differentiation in mature oligodendrocytes or the proliferation and maturation of pre-existing precursor cells. Activation of these pathways is believed to involve the complement system and growth factors or cytokines respectively (Fig. 9.4). However, widespread remyelination in demyelinated plaques is not often found. This may be due to inhibition of remyelination by the action of activated astrocytes, macrophages, and lymphocytes (Fig. 9.5). These cells attack myelin or oligodendrocytes or in the case of astrocytes, form physical barriers by surrounding nude axons and thereby preventing remyelination (Ludwin, 1995). These cells have been implicated in promoting myelin degradation in our mechanism of demyelination.

The central reactions in our mechanism of demyelination include modification of PLP and DM20 by hyper fatty acylation, the activity of PAD on MBP which gives rise to increased levels of MBP-Cit6 and myelin instability, the degradation of MBP and the BBB by MMPs, the role of activated astrocyte in attacking myelin and oligodendrocytes, and the autoimmune response by lymphocytes. It was postulated that if these reactions can be targeted simultaneously, we may be able to inhibit the progression of disease and promote remyelination.
Figure 9.4: Potential mechanism of remyelination. Remyelination may occur through de-differentiation of oligodendrocyte from survived cells or from unaffected areas around lesions. Alternatively, stem cells originating from the ependymal layer of the lateral ventricles of the brain may be stimulated by growth factors and cytokines to proliferate and mature into myelinating oligodendrocytes.
Figure 9.5: Reactions that inhibit remyelination. Remyelination is inhibited by attacking oligodendrocytes or oligodendrocyte precursor cells through the action of astrocytes, macrophages, and lymphocytes. Formation of physical barriers around nude axons by astrocyte processes block access of myelinating cells to axons thereby preventing remyelination.

Paclitaxel is commonly known for its antimitotic properties by inhibiting microtubule depolymerization (Schiff et al., 1979). More recently, it has been shown to inhibit MMP expression in vitro (Hui et al., 1998), to activate interleukin-8 (IL-8) gene expression (Lee et al., 1996), and to inhibit the activity of PAD in vitro (Pritzker and
Moscarello, 1998). Therefore, paclitaxel may be capable of targeting different players involved in the mechanism of demyelination. Using the ND4 transgenic mice as a model for demyelinating disease, the effectiveness of paclitaxel as a treatment for demyelinating disease was tested by injecting the drug into affected animals. Observation of the clinical progression in paclitaxel treated ND4 mice revealed an attenuation of disease by the drug (Fig. 6.2). The severity of clinical signs were reduced further when treatment was started prior to onset of clinical signs. This reduction was maintained two months after the last injection of paclitaxel indicating the effect of this drug can be sustained for lengthy periods.

Morphological examination of treated transgenic mice demonstrated that paclitaxel promoted remyelination. This was observed by increased number of myelinated axons and fewer nude axons in light microscopy sections of both the posterior column of the spinal cord and the superior cerebellar peduncle (Figs. 8.1 and 8.2). An increase in myelinated fibers and thinly myelinated axons, the hallmark of remyelination, was also observed under EM in sections of brain and optic nerve from treated ND4 mice (Figs. 8.3 and 8.4). The promotion of remyelination by paclitaxel may be due to the reduction of proliferating astrocytes. By removing these cells, paclitaxel would eliminate one of the players involved in the attack on myelin and oligodendrocytes and also the physical barrier formed by astrocyte processes which block access to axons (Ludwin, 1995). The inhibition of proliferating astrocytes in ND4 mice by paclitaxel was observed by the reduction in the number of GFAP stained cells in light microscopy brain sections and reduced levels of GFAP in whole brain homogenate (Table 6.1). Electron micrographs of sections from treated ND4 mice showing disrupted microtubules and rounded nuclei with condensed chromatin within astrocytes indicated that these cells were dying. This was also suggested in GFAP stained light microscopy sections where GFAP stained halos appeared around astrocytes. Since these characteristics were not observed in paclitaxel treated normal mice, these findings suggested that proliferating astrocytes in demyelinating animals were
targeted and inhibited by paclitaxel. These results demonstrate that paclitaxel is an effective therapeutic drug for the treatment of demyelinating disease.

The effectiveness of paclitaxel may not be due entirely to the inhibition of proliferating astrocytes. Paclitaxel may affect other targets in the mechanism of demyelination (Fig. 9.6). Since paclitaxel inhibits PAD activity in vitro (Pritzker and Moscarello, 1998), it is likely that this drug may have the same effect in the ND4 mice. Results from a preliminary study demonstrated that PAD activity was decreased to normal levels in 6 month old ND4 mice after treatment with paclitaxel (Mak, unpublished). By inhibiting PAD activity, the modification of MBP-Cit0 into MBP-Cit6 should also be inhibited. The expected results could be a more stable myelin and reduced incidence of demyelination. A more extensive study on PAD activity in paclitaxel treated ND4 mice will need to be performed to verify these findings.

Paclitaxel may also affect the proliferation of lymphocytes. This was demonstrated by Whitaker et al. using two different methods (Whitaker, unpublished). In one set of experiments, these investigators found that cultured lymphocytes sensitized to MBP failed to incorporate $^3$H-thymidine upon exposure to paclitaxel. In another set of experiments, Whitaker et al. found that EAE induced by passive transfer was eliminated in mice that were also injected with paclitaxel (Whitaker, manuscript in preparation). These results demonstrated paclitaxel's ability to inhibit the proliferation of lymphocytes reactive towards MBP and the immune response induced by encephalitogenic antigens. In this manner, paclitaxel may be able to block the autoimmune response in the mechanism of demyelination. Another potential target for paclitaxel could be the expression of MMPs. However, treatment of ND4 mice did not decrease the expression levels of stromelysin-1 mRNA (Fig. 7.8). Since MMPs are required for tissue remodeling, it is likely that expression of this MMP is required during remyelination. A more in-depth study on the
Figure 9.6: Potential targets of Paclitaxel. Paclitaxel inhibits proliferating cells such as astrocytes and lymphocytes in animal models of demyelination. It has also been found to decrease PAD activity in vitro and the activity of MMPs. Inhibition of MMPs could reduce the digestion of MBP and components of the blood brain barrier.
involvement of specific MMPs in demyelination is currently underway in our laboratory. Identification of individual MMPs will allow us to examine changes in their expression and activity during stages of disease and after paclitaxel treatment. By inhibiting the proliferation of astrocytes, the activity of PAD, the proliferation and immune response of lymphocytes, and perhaps the expression of specific MMPs, paclitaxel is capable of targeting different players in the mechanism of demyelination at the same time. In this manner, paclitaxel may have attenuated clinical severity of disease and promoted remyelination in the ND4 mice.

The findings reported in our study using the ND4 transgenic mouse lead to a phase-1 study of paclitaxel in the treatment of secondary progressive MS. In this study, 30 patients were treated with 25 or 50 mg/M² paclitaxel monthly for a total of six treatments and evaluated clinically and by MRI throughout treatment. Favorable trends were observed in both MRI and clinical parameters for patients treated with paclitaxel compared with a placebo group (O'Connor, abstract submitted to the American Neurological association meeting, 1999). Thus paclitaxel is an effective drug treatment for demyelinating disease.

**Concluding remarks**

Using a spontaneously demyelinating animal model, the reactions which may be responsible for the disruption of myelin were elucidated from which a mechanism of demyelination was proposed. This mechanism was based on changes in the post-translational modification of the myelin proteins MBP and PLP/DM20. These changes may form the primary defects inherent in oligodendrocytes which could alter the stability of the myelin membrane and increase its susceptibility to attack. Reaction to a less stable myelin by extracellular components and other cells may result in the induction of an autoimmune response characteristic of demyelinating disease. Thus the immune system participates as a secondary reaction in the mechanism of demyelination. This conclusion is supported by
the inconsistent presence of inflammatory cells and T-cells in areas of demyelination (Rodriguez and Scheithauser, 1994), the degeneration of myelin in areas outside of maximum inflammation (Rodriguez and Scheithauser, 1994), the uniform degeneration of areas inaccessible to direct immune attacks such as the inner loops of myelin (Dawson, 1916; Rodriguez and Scheithauser, 1994), and the absence of increased MBP-Cit6 in EAE animals, a model for the immunological response in demyelinating disease (Mastronardi et al., 1996a). In summary, the proposed mechanism of demyelination can be divided into three parts beginning with a defect in oligodendrocytes such as changes in the post-translational modification of PLP and DM20. This is followed by enzymatic degradation or modification of MBP in myelin which may result in the response and activation of astrocytes, macrophages, lymphocytes, cytokines, and MMPs. Thus demyelinating disease can be viewed as a post-translational modification disease of myelin proteins.

With this mechanism of demyelination in mind, treatment of ND4 mice using paclitaxel attenuated clinical progression of disease and promoted remyelination. Paclitaxel functioned in a multi-targeting manner by inhibiting proliferating astrocytes, lymphocyte activation, and PAD activity. It may also inhibit the digestion of MBP and the degradation of the BBB by specific MMPs. Thus paclitaxel was successful in the ND4 transgenic mice due to the targeting of several central players proposed in the mechanism of demyelination. These results suggest that paclitaxel is an effective treatment for experimental demyelinating disease.
Future Studies

1) The low levels of PLP in ND4 mice remains unresolved despite the studies at the transcription factor and mRNA levels. To determine the defect resulting in low PLP protein levels, future experiments should include:
   a. Transcription rate measurements for the PLP gene and the DM20 transgene.
   b. Translation rate experiments for PLP mRNA, endogenous DM20 mRNA, and transgene DM20 mRNA to determine if the translation of PLP protein in reduced.
   c. Determining the subcellular localization of the hyperacylated PLP and DM20 proteins in oligodendrocytes from ND4 mice to detect changes in the cellular distribution of these proteins.

2) In our hypothesis, it was proposed that the high DM20 mRNA or protein levels maintained oligodendrocytes in a developmentally immature stage. To demonstrate that oligodendrocytes in ND4 mice are arrested at an earlier stage of development, brain sections from adult transgenic mice will be stained with cell markers specific to oligodendrocyte at different stages of development such as the A2B5 ganglioside marker for developmentally immature oligodendrocytes (Eisenbarth et al., 1979; Kasai and Yu, 1983) and Gal C for mature oligodendrocytes (Sommer and Schachner, 1981). If cells are developmentally immature, an increase in staining for markers for early development is expected in white matter tracts from transgenic mice.

3) Hyperacylation of PLP and DM20 may also be a natural modification in less developed oligodendrocytes. Using neo-natal mouse brain from at least 20 mice, DM20 and PLP will be isolated and then separated using a CM tris-acryl column. Covalently bound fatty acids from the proteins will be analyzed by GC/MS to measure the levels of
palmitate and stearate. If hyperacylation of these proteins occurs naturally in a less
developed oligodendrocyte, increased levels of these fatty acids is expected.

4) To demonstrate that developmentally immature oligodendrocytes are
characteristic of demyelinating disease, the relative amounts of PLP and DM20 protein will
be measured in white matter from MS patients. This will be performed using supernatant
from white matter homogenate by the immunoslot blot method (Barrese et al., 1998). If
oligodendrocytes in MS are less developed, high DM20 and low PLP levels are expected.

5) Since the ND4 mice were used as a model for demyelinating disease, the
hyperacylation of PLP and DM20 may be restricted to these animals. The occurrence of
this modification during demyelination in humans should be determined. In a future
experiment PLP and DM20 will be isolated from white matter from MS patients and
analyzed for changes in the amount of covalently bound fatty acids.

6) Increased affinity for lipids in a bilayer environment by hyperacylated
PLP/DM20 was postulated to alter the activity of PAD. This relationship will be studied by
incorporating modified PLP/DM20 into DPPC vesicles along with purified PAD from
bovine brain. The activity of PAD within the vesicles will be assayed and compared to
values obtained from vesicles containing unmodified PLP/DM20. An increase in PAD
activity in vitro would suggest that the modified PLP/DM20 may have a similar effect in
myelin.

7) Future experiments on the effects of paclitaxel in ND4 mice should include:

a) The study of paclitaxel on the levels of covalently bound fatty acids in PLP and
   DM20 protein. This will be examined by isolating PLP and DM20 protein from
at least 20 ND4 mice treated with paclitaxel and analyzing bound fatty acids by GC/MS to determine if the treatment reduces the amount of covalently bound fatty acid.

b) Confirmation of the inhibition of PAD activity using a larger group of animals studied at different ages after injection of the drug. This experiment will provide insight into the change in PAD activity as the animals recover. Also, the effect of paclitaxel on changes in MBP charge isomers should also be included.

c) Examination of lymphocyte proliferation and response in treated ND4 mice.

d) Identification of the specific MMPs involved in the process of demyelination in the ND4 mice and determining the effect of paclitaxel on their expression and activity.
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