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Cotranslational Integration of Myelin Proteolipid Protein (PLP) Into the Membrane of Endoplasmic Reticulum: Analysis of Topology by Glycosylation Scanning and Protease Domain Protection Assay

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ABSTRACT The four transmembrane domain topology of the proteolipid protein (PLP) in the myelin membrane of the central nervous system (CNS) has been further substantiated by biochemical studies. We have analyzed the cotranslational polytopic integration of nascent PLP during protein synthesis into the membrane of the endoplasmic reticulum (ER) on two routes. Consensus sequences for N-glycosylation were introduced by site directed mutagenesis into the PLP sequence as reporter sites, which upon glycosylation monitor the intraluminal location of the respective domains corresponding to the extracellular side of the plasma membrane. Single, double, and triple mutant cDNAs were constructed for transcription/translation in vitro in the presence of ER-membranes. The glycosylation pattern of the translation products revealed that hydrophilic extramembrane regions 2 and 4 (EMR2/EMR4) and EMR3 of PLP are exposed on opposite sides of the ER membrane. Their localization either at the cytosolic or luminal side of the ER membrane leads to two different topologies. The two modes of membrane integration during in vitro cotranslational translocation were confirmed by protease protection assays with wild-type and truncated PLP polypeptides with either one, two, or three putative transmembrane domains integrated into the ER-membrane. The fragment pattern of the [^{35}S]methionine- or [^{3}H]leucine-labeled polypeptides revealed that EMR3 and EMR4 were exposed with opposite orientation either on the cytosolic or luminal side of the ER membrane supporting the 4-transmembrane helix (TMH) N_{in} model with the N and C termini on the cytoplasmic side, as established for the myelin membrane (plasma membrane); the other inversely integrated PLP constructs indicate the 4-TMH-N*out* profile. These results are discussed with regard to the PLP biogenesis and the plasma membrane topology in PLP-expressing cells. *GLIA 24:226–235, 1998.*

INTRODUCTION

Myelin is a highly compacted multilamellar membrane system, which ensheathes the nerve axons between the nodes of Ranvier and permits fast saltatory conduction of nerve impulses. In the central nervous system (CNS) myelin sheaths are formed from plasma membrane processes of oligodendrocytes. Two major

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constituents in the limited repertoire of myelin proteins are believed to be essential for myelin compaction. Myelin basic proteins (MBPs) are peripheral membrane proteins (Eylar, 1970; Kamholz et al., 1986; Takahashi et al., 1985) that are attached to the cytoplasmic surface and contribute to the tight adhesion of the cytoplasmic membrane surfaces (Martini et al., 1995; Readhead et al., 1987). Proteolipid isoproteins, PLPs, and their splice variant DM20, are integral membrane proteins.

The amino acid sequence of the hydrophobic PLP is extremely conserved among mammalian and phylogenetically distant species (Schliess and Stoffel, 1991). The importance of PLP in the organization and stabilization of the myelin sheath is obvious from several naturally occurring mutations in different species including man. These are mostly point mutations within the PLP gene or RNA splice defects with the phenotype of a severe dysmyelinosis due to premature apoptosis of the oligodendrocytes during the myelination period and hypomyelination. Ablation of PLP gene expression by the gene targeting approach in mice causes disruption of the apposition of the myelin sheath at the outer surface visualized by the loss of the intraperiod dense line (IDL) in electron microscopy. The tight apposition of the cytoplasmic membrane surfaces (main dense line, MDL) remained unimpaired (Boison and Stoffel, 1994; Boison et al., 1995; Klugmann et al., 1997).

Different experimental strategies have been applied to determine the topography of PLP integrated into the specialized lipid bilayer synthesized by oligodendrocytes during myelinogenesis: photoaffinity labeling (Kahan and Moscarello, 1985), X-ray diffraction (Inouye and Kirschner, 1989), trypsin treatment of hyposmotically shocked myelin and subsequent analysis of protein fragments (Stoffel et al., 1984), and immunocytochemical epitop mapping (Hudson et al., 1989; Stoffel et al., 1989).

The ambiguity of these models was resolved by the determination of the functional state and the sidedness of the 14 cysteine residues of PLP in the free-SH-, disulfide-, or thioacylated form. A 4 TMH model of PLP has been derived from these biochemical studies. Four clusters of hydrophobic amino acid residues are aligned as membrane spanning sequences. They are connected by two extracytoplasmic and three cytoplasmic domains, including the N and C termini (Weimbs and Stoffel, 1992, 1994) (see Fig. 1). This topology of PLP agrees with the 4 TMH-N_{in} model with the N and C termini on the cytoplasmic side one of the two proposed theoretical models (Popot et al., 1991). Recent immunocytochemical studies on cultured primary oligodendrocytes using polyclonal and monoclonal antibodies that recognize particular PLP sequences confirm the extracytoplasmic orientation of two hydrophilic loops (Gow et al., 1997; Greer et al., 1996) and the cytosolic localization of the C terminus and the positively charged sequence that is deleted in the DM20-isoform (Gow et al., 1997; Konola et al., 1992; Sobel et al., 1994).

In the present study, we examined the translational translocation of PLP into the ER membrane during synthesis. The 4 TMH topology in the ER membrane was substantiated further by two independent biochemical approaches: a) N-glycosylation consensus sequences (N-X-T/S; Hart et al., 1978) were introduced by site directed mutagenesis into the hydrophilic extramembrane regions, which connect the four transmembrane domains of PLP. The cDNA constructs were transcribed and translated in vitro in the presence of canine microsomal membranes. The degree and the pattern of the N-glycosylation after in vitro insertion of the mutant PLP polypeptides into microsomal membranes monitored the cytosolic or luminal orientation of the glycosylation sites. b) In a second approach full length and truncated PLP polypeptides were synthesized in vitro and integrated into microsomal membranes. Protease treatment of the intact ER membranes and the analysis of the fragments allowed the assignment of the domains exposed to proteolysis.

We conclude from these in vitro studies that the loop connecting transmembrane domains (TMD) II and III of PLP is exposed on one side and the two sequences connecting TMDI/TMDII and TMDIII/IV are localized on the opposite surface of the ER membrane. The observation that inserted PLP spans the microsomal membranes in two inversed orientations is discussed.

MATERIALS AND METHODS

cDNA Constructs

All PLP constructs used in this study were derived from a pUC13 vector, which contained the full length PLP cDNA including an ~2 kb 3′-untranslated region described before (Schaich et al., 1986). A 5′sense oligonucleotide containing a Hind III restriction site, the start codon ATG, and the ideal Kozak sequence was synthesized for amplification of the coding region of PLP by PCR. The 3' antisense oligonucleotide contained the stop codon TAG and a XbaI site. The amplified cDNA was cloned in antisense orientation into pGEM3Z under the control of the SP6-promotor (pGEM-PLP). This vector was used as template for site directed mutagenesis. Single N-glycosylation consensus sequences N-X-T or N-X-S (Hart et al., 1978) were introduced into the PLP cDNA at position 139, 339, and 579 by overlapping extension (SOE) PCR (Higuchi et al., 1988). cDNA cassettes with the substitution mutations N46I and N113S were released by HindIII/BglII-, the cassette with the substitution mutation N193S by BglII/NcoI digestion. They were exchanged against the respective restriction fragments of wild-type pGEM-PLP. The double mutant cDNAs pGEM-N46I/N193S and pGEM-N113S/N193S were synthesized by insertion of HindIII/BglII fragments from the respective single mutants into construct pGEM-N193S. The N46I/N113S mutation was introduced by additional SOE-PCR using pGEM-N46I as template DNA. The triple mutant was created by substitution of the BglII/NcoI fragment of the double mutant pGEM-N46I/N113S by the BglII/NcoI fragment from pGEM-N193S.

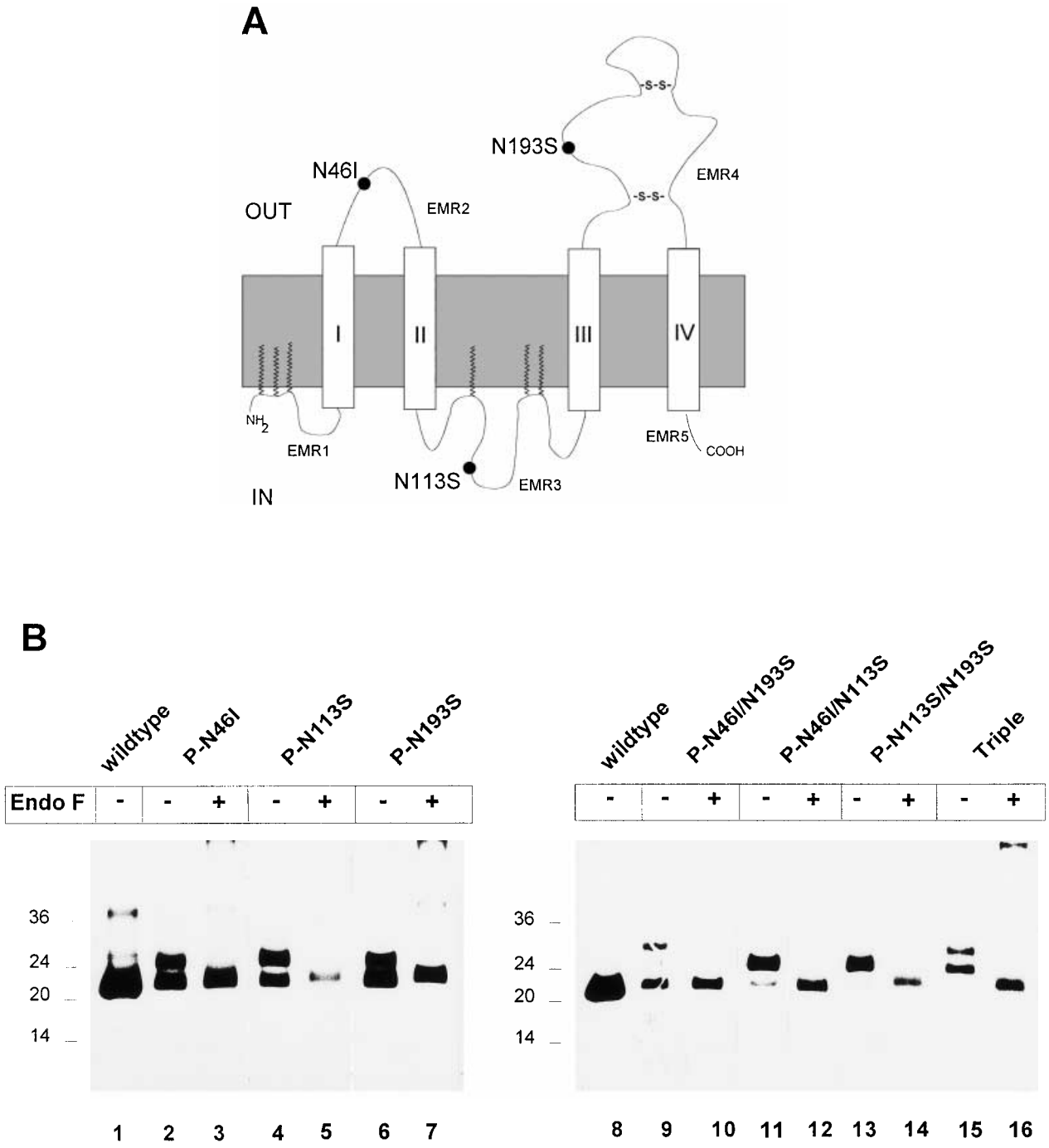

Fig. 1. **A:** 4 TMH-N_{in} topology of the proteolipid protein. Four hydrophobic domains (TMD I–IV) span the myelin membrane bilayer. The N and C termini, as well as the six acylated cysteine residues (indicated as zigzag lines), are located at the cytoplasmic membrane surface. The two disulfide bridges in the hydrophilic loop connecting TMD III and TMD IV are placed at the extracellular surface (Weimbs and Stoffel, 1992). N-glycosylation sites (N-X-T/S; Hart et al., 1978) introduced by site directed mutagenesis are indicated by black dots. **B:** N-glycosylation of mutant PLP containing one, two, or three consensus sequences N46I, N113S, and N193S. Mutagenized cRNAs were translated in the rabbit reticulocyte lysate in the presence of canine pancreas microsomes. [^{35}S]Methionine-labeled translation products were isolated by ultracentrifugation of the microsomal membranes. Proteins were separated by SDS-PAGE (12%) and visualized by fluorography. Molecular mass of PLP mutants modified by one oligosaccharide chain decreased by 3 kDa and those at two consensus sites by 6 kDa after digestion with endoglycosidase F. Except in lanes 11, 13, and 15, approximately equal amounts of unglycosylated translation products are visible.

Truncated cDNAs with stop codon TAG at positions 178 (E59; PLP-I), 478 (L159; PLP-II), or 709 (T236; PLP-III) were generated by PCR using pGEM-PLP as template DNA. Amplified fragments were digested with HindIII and XbaI and cloned into pGEM3Z. All subcloned cDNAs were sequenced in both directions between the sites of the indicated restriction enzymes.

For transcription of the apolipoprotein AI cDNA, the plasmid pDS5-AI was used (Stoffel and Binczek, 1988).

RNA Transcription

Plasmids were linearized with BamHI. The respective methyl-diguanosine-triphosphate-capped cRNAs were synthesized from their corresponding linearized templates using SP6 RNA polymerase (Boehringer Mannheim, Germany) following standard procedures (Colman, 1984). RNA was controlled by electrophoresis in formaldehyde (1.2%) agarose gel.

Cell-Free Translation and Deglycosylation

cRNA (300–500 ng) was translated in micrococcal nuclease treated rabbit reticulocyte lysate (amino acid depleted; Amersham Corp., Braunschweig, Germany) in the presence of canine pancreatic rough microsomal membranes (Boehringer Mannheim, Germany) according to the supplier's instructions. The reaction mixture contained in a total volume of 30 µl: 1,110 kBq [^{35}S]methionine or 1850 kBq [^{3}H]leucine (Amersham Corp., Braunschweig, Germany), 25 µM of each amino acid except methionine (or leucine), 100 mM potassium acetate, and 2 mM magnesium acetate and was incubated at 30°C for 60 min;

30 µl STPM buffer (0.25 M sucrose, 50 mM triethanolamine, 140 µmM potassium acetate, and 2.5 µmM magnesium acetate) was added and membranes were sedimented by ultracentrifugation for 10 min (4°C; 45,000 rpm) and the pellet rinsed with 50 µl STPM. One aliquot was resuspended in 50 µl Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% mercaptoethanol, 1% SDS, and 0.04% bromophenol blue] for SDS-PAGE and the other in 25 µl 0.75% Triton X-100, 30 mM Tris-HCl (pH 8) for deglycosylation with endoglycosidase F (50 mU) (Boehringer Mannheim, Germany) for 1 h at 37°C. The reaction was terminated by the addition of 25 µl 2× Laemmli sample buffer and analyzed by SDS-PAGE (Laemmli, 1970) followed by fluorography (Laskey and Mills, 1975).

Alkaline Extraction of Membranes

The pH of the in vitro translation assay, carried out in the presence of rough microsomal membranes, was adjusted to 11.5 by the addition of 25 µl of carbonate buffer [50 mM potassium acetate, 100 mM Na_2CO_3, 20 mM triethanolamine (TEA), and 1 mM magnesium acetate). The reaction mixture was stored on ice for 20 min and layered on top of 20 µl STPM containing 100 mM Na_2CO_3. Microsomes were sedimented at 49,000 rpm at 4°C for 20 min. The pellet was washed with 25 µl STPM and dissolved in 30 µl Laemmli sample buffer. The supernatant was neutralized with 10 µl acetic acid. Proteins were precipitated with 2 vol of saturated ammonium sulfate, incubated for 30 min at 0°C, and sedimented at 14,000 rpm, 4°C for 10 min. The pellet was washed with 5% trichloroacetic acid and dissolved in 50 µl Laemmli loading buffer for SDS-PAGE.

Proteinase K Digestion

Immediately following the in vitro translation reaction (total volume 30 µl), 18 µl of the reaction mixture was mixed with 18 µl STPM buffer, cooled on ice and the $CaCl_2$ concentration adjusted to 10 mM. Microsomal membranes of 12 µl and 24 µl aliquots were sedimented by ultracentrifugation (10 min, 4°C, 45,000 rpm). The pellet of the 12 µl aliquot was dissolved in 50 µl Laemmli sample buffer for control of the translation efficiency. The membranes of the 24 µl aliquot were resuspended in 25 µl TPM (50 mM triethanolamine, 140 mM potassium acetate, and 2.5 mM magnesium acetate), placed on ice and treated with 1.5 µl proteinase K (1 mg/ml) in the presence of 1% Triton X-100 for 60 min at 0°C. Subsequently, the reaction was terminated by addition of 25 µl 2× Laemmli sample buffer. To the remaining aliquot of the translation reaction 12 µl TPM and 1.5 µl proteinase K was added in the absence of the detergent. After incubation on ice for 60 min, proteolysis was arrested by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2.5 mM. Membrane integrated proteolytic fragments were isolated by ultracentrifugation (see above) and dissolved in 50 µl Laemmli sample buffer. Proteins and proteolytic fragments were analyzed by SDS-PAGE followed by fluorography.

RESULTS

N-Glycosylation of Extramembrane Regions Flanking Putative Hydrophobic Domains

We examined the topology of the extramembrane regions EMR2, 3, and 4 that link the four transmembrane domains TMD I-IV of the PLP polypeptide by N-glycosylation scanning (Fig. 1A). The insertion of PLP into ER-membranes was studied in the reticulocyte in vitro synthesis system supplemented with canine microsomal membranes. The N-glycosylation is restricted to the luminal compartment of the endoplasmic reticulum. Therefore only consensus sites of polypeptides which are exposed at the luminal side are modified. They will be exposed at the outer surface of the plasma membrane after protein trafficking and sorting.

Native PLP is unglycosylated. Therefore consensus signals for N-glycosylation (N-X-T/S) were introduced into EMR2 (N46I), 3 (N113S), and 4 (N193S) by site directed mutagenesis (Fig. 1A). The cRNA transcripts of mono, double and triple mutants were translated in vitro using the [^{35}S]methionine-supplemented reticulocyte lysates and rough microsomes. Translation products translocated into the microsomal membranes were isolated by ultracentrifugation and analyzed by SDS-PAGE with subsequent fluorography. The single substitution mutants P-N46I and P-N193S were monoglyco-

sylated. Enzymatic deglycosylation by endoglycosidase F (EndoF) reduced their molecular mass by 3 kDa (Fig. 1B, lanes 2 and 3, 6 and 7). Therefore the two sites should be exposed at the luminal face of the ER membrane, which is in support of the localization of EMR2 and EMR4 at the outer surface of the plasma membrane. Surprisingly, nearly an equal amount of polypeptides remained unglycosylated, which indicated the cytoplasmic orientation of the glycosylation tag (Fig. 1B, lanes 2 and 6). Unmodified and monoglycosylated polypeptides were obtained from the mutant P-N113S (Fig. 1B, lanes 4 and 5). This challenged the proposed cytoplasmic orientation of EMR3.

Posttranslational N-glycosylation of the double mutants P-N46I/N113S and P-N113S/N193S yielded monoantennary glycosylated polypeptides as the main translation products (Fig. 1B, lanes 11–14). Therefore the mutagenized residue S113 (N113S) on one hand and residues I46 (N46I) and S193 (N113S) on the other must be oriented toward opposite sides of the membrane. Nearly equal amounts of the unglycosylated and of the twofold glycosylated form of the double mutant P-N46I/N193S were synthesized differing approximately by 6 kDa in size , equivalent to two N-oligosaccharide groups (Fig. 1B, lanes 9 and 10). Therefore asparagine residues N46 and N193 had the same sidedness.

The N-glycosylation pattern of the triple mutant gave equal signal intensities of one- and twofold glycosylated polypeptides. In contrast to the single mutants only negligible amounts of unmodified protein were detectable (Fig. 1B, lanes 15 and 16). However, these data do not allow the assignment of the individual N-glycosylated domain to the luminal or cytoplasmic compartment. The nascent PLP chain is integrated into the microsomal membrane with the N and C termini positioned at the cytoplasmic side and thereby in accordance with the 4 TMH-N_{in} model derived from previous topological experiments (Weimbs and Stoffel, 1992) (see Fig. 1A), or with the N and C termini at the luminal side of the ER membrane, which would correlate with the inverse oriented 4 TMH-N_{out} model (Popot et al., 1991).

Protein Domain Protection Assay of Wild-Type and Truncated PLP-Protein

We have chosen a second approach for the topological mapping of PLP domains. Protease treatment of PLP will cleave domains located on the outer (cytoplasmic) side of the microsomal vesicles, whereas luminally oriented domains remain protected. Wild-type and truncated translocated PLP polypeptides containing either one, two, or three putative transmembrane domains were differentially labeled with [^{35}S]methionine and [^{3}H]leucine and subsequently membrane integrated polypeptides treated with proteinase K. The proteolytic fragments were assigned to the N-or C-terminal part of membrane integrated PLP.

Essential for this protection assay are the i) integrity of the microsomal membranes and ii) membrane integration of the translated PLP-polypeptides. The tightness of the vesicular membranes was assayed by in vitro translation and translocation of the cRNA of the secretory preproapolipoprotein AI (267 amino acids). Processed proapo AI was translocated into the lumen of the vesicles after cleavage of the 18 amino acid signal sequence (lower band) and was therefore protected against proteinase K proteolysis, whereas the unprocessed protein (upper band) was completely degraded (Stoffel et al., 1988) (Fig. 2A, lanes 1 and 2). Permeabilization of the membrane with the non-ionic detergent triton X-100 led to complete proteolysis of the translation product (Fig. 2A, lane 3).

Integral membrane proteins can only be solubilized by detergent treatment of the membrane. Alkali treatment removes only peripheral membrane and secretory proteins without disrupting the lipid bilayer with its integral membrane proteins (Fujiki et al., 1982; Russel and Model, 1982). The canine microsomal membranes with the integrated newly synthesized PLP polypeptides were treated with sodium carbonate (pH 11.5) and centrifuged through a sucrose cushion. Whereas secretory proapo AI was released into the supernatant (Fig. 2B, lane 10), wild-type and truncated PLP-polypeptides sedimented with the membranes (Fig. 2B, lanes 1, 3, 5, 7).

The most N-terminal methionine of the 276 residue long mature translation product of cRNA of PLP is at position 206. Therefore only full length PLP and truncated PLP-III, which contains putative transmembrane domains I–III, were labeled with [^{35}S]methionine (Fig. 3B, lanes 1 and 4). Constructs PLP-I and PLP-II remained unlabeled (data not shown). This is in agreement with previous results that showed that in vitro synthesized PLP is processed by cleavage of the N-terminal methionine (Colman et al., 1982). Proteinase K digestion of PLP with subsequent recovery of the membrane embedded, [^{35}S]methionine labeled proteolytic fragments by ultracentrifugation yielded only fragments of size 12–13 kDa (F-III/C1) from PLP-III (Fig. 3B, lane 2) and two polypeptides derived from full length PLP with an apparent molecular mass of 16–17 kDa (F-WT/C1) and ~6,5 kDa (F-WT/C2), respectively (Fig. 3B, lane 5). The three fragments originate from the C-terminal half of the PLP polypeptides, because i) the first methionine is located at position 206 and ii) the 5 kDa difference in size of fragments F-III/C1 and F-WT/C1 reflects that of PLP-III and wild type. Protease treatment releases the 6,5 kDa [^{35}S]methionine labeled fragment F-WT/C2 only from full length PLP but not from PLP-III. Therefore this fragment represents the C terminus of PLP.

In addition, [^{3}H]leucine labeling of PLP yielded proteolytic fragments of sizes 19–20 kDa (F-WT/N2; F-III/N2) and ~10 kDa (F-WT/N1; F-III/N1) (Fig. 3A, lanes 8 and 11). The ~10 kDa fragment was also released from the truncated PLP-II (F-II/N1) in the protease protection assay (Fig. 3A, lane 5). The ~10 kDa fragments F-WT/N1; F-III/N1 and F-II/N1 therefore represent sequences derived from the N-terminal 159 amino acids of PLP. The 6,5 kDa polypeptide PLP-I, which contains

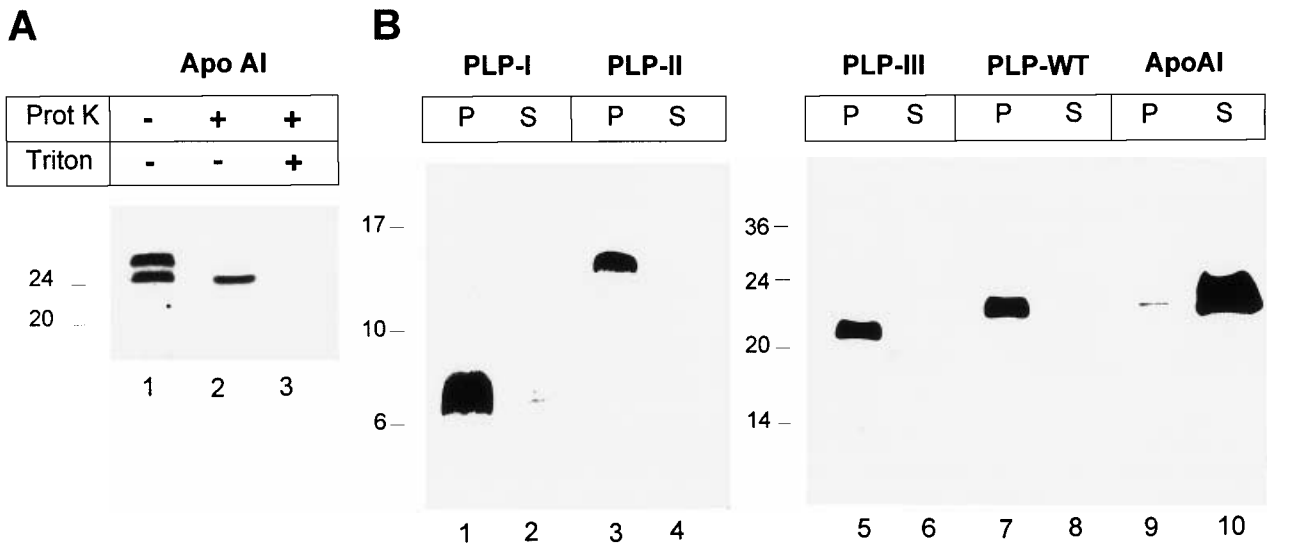

Fig. 2. **A:** Protease protection assay reveals integrity of microsomal membranes. ApoAI cRNA was translated in vitro with rough dog pancreas microsomes as described in Figure 2. Translation products were digested with proteinase K in the presence (+) or absence (−) of Triton X-100 and subsequently analyzed by SDS-PAGE and fluorography. In the control (lane 2) preproApoAI not translocated across the ER membrane (upper band, lane 1) was degraded, whereas proapoAI, processed and residing in the ER lumen, was protected against proteolysis (lower band, lane 1). **B:** Membrane integration of wild-type and truncated PLP polypeptides. In vitro translation reactions supplemented with rough microsomes were extracted with sodium carbonate at pH 11.5 and membranes sedimented by centrifugation through a sucrose cushion. Truncated proteins PLP-I, PLP-II, and PLP-III with one, two, or three hydrophobic TMD-domains respectively, and wild-type PLP were recovered in the pellets (P) (lanes 1, 3, 5, and 7). In the control, translation products of ApoAI cRNA were found in the supernatant (lane 10).

only the first putative transmembrane domain, was apparently resistant against proteinase K digestion (Fig. 3A, lane 2). Protease treatment in the presence of the detergent Triton X-100, however, led to complete degradation of all translation products (Fig. 3A, lanes 3, 6, 9, 12).

From the size of the proteolytic fragments it is reasonable to conclude that two extramembrane regions of PLP are accessible for proteinase K. Cleavage in EMR3 of the wild-type protein released F-WT/N1 (~10 kDa) and F-WT/C1 (16–17 kDa), and of the truncated PLP-III yielded F-III/N1 (~10 kDa) and F-III/C1 (12–13 kDa). The apparent molecular mass of PLP-II was reduced by ~6 kDa (see Fig. 3A,C). Also EMR4 is protease sensitive, as indicated by the release of proteolytic fragments F-WT/N2 (19–20 kDa) and F-WT/C2 (6,5 kDa) from PLP (Fig. 3A,D). PLP-III was shortened by 3–4 kDa. No proteolytic cleavage occurred in EMR2.

DISCUSSION

X-ray crystallographic information on the topology of PLP are not yet available. Therefore various biochemical and immunocytochemical strategies have been applied to deduce the membrane topology from the sidedness of protein domains of the mature form of PLP in the CNS myelin lamellae or plasma membrane of PLP expressing cells. The results of these studies were however inconclusive (Gow et al., 1997; Greer et al., 1996; Hudson et al., 1989; Konola et al., 1992; Sobel et al., 1994; Stoffel et al., 1989). The hydropathy plot of PLP suggested four hydrophobic transmembrane domains, which are linked by three hydrophilic extramembrane domains. A 4 TMH-N_{in}-model has been experimentally substantiated by using differential labeling of the 14 cysteine residues of PLP with a thiol-specific fluorescent probe to determine their functional state (free -SH, -S-S-, and thioesterified) and by vectorial labeling of lysine residues on the outer surface of myelin (Weimbs and Stoffel, 1992, 1994).

PLP is synthesized in the rough endoplasmic reticulum of oligodendrocytes and transfected cells. It is reasonable to assume that PLP acquires its final three-dimensional structure during ribosome bound synthesis and translocation into the ER membrane (Gow et al., 1994; Nussbaum and Roussel, 1983; Schwob et al. 1985, Sinoway et al., 1994).

The reticulocyte lysate/rough microsome expression system has been well established by numerous in vitro topological studies. We applied this system to studies on the topology of nascent PLP, translocated into the ER membrane for sorting to the plasma membrane. In the first approach we probed the sidedness of the extramembrane sequences linking the four putative transmembrane domains by N-glycosylation scanning. Single N-glycosylation consensus sequences were introduced at residues of the putative extramembrane domains (EMR) by site-directed mutagenesis: N46I within hydrophilic EMR2, N113S within EMR3, and N193S probing EMR4 of wild-type PLP. Mutant PLPs with two or three consensus signals at these sites in all possible combinations were constructed to probe the orientation

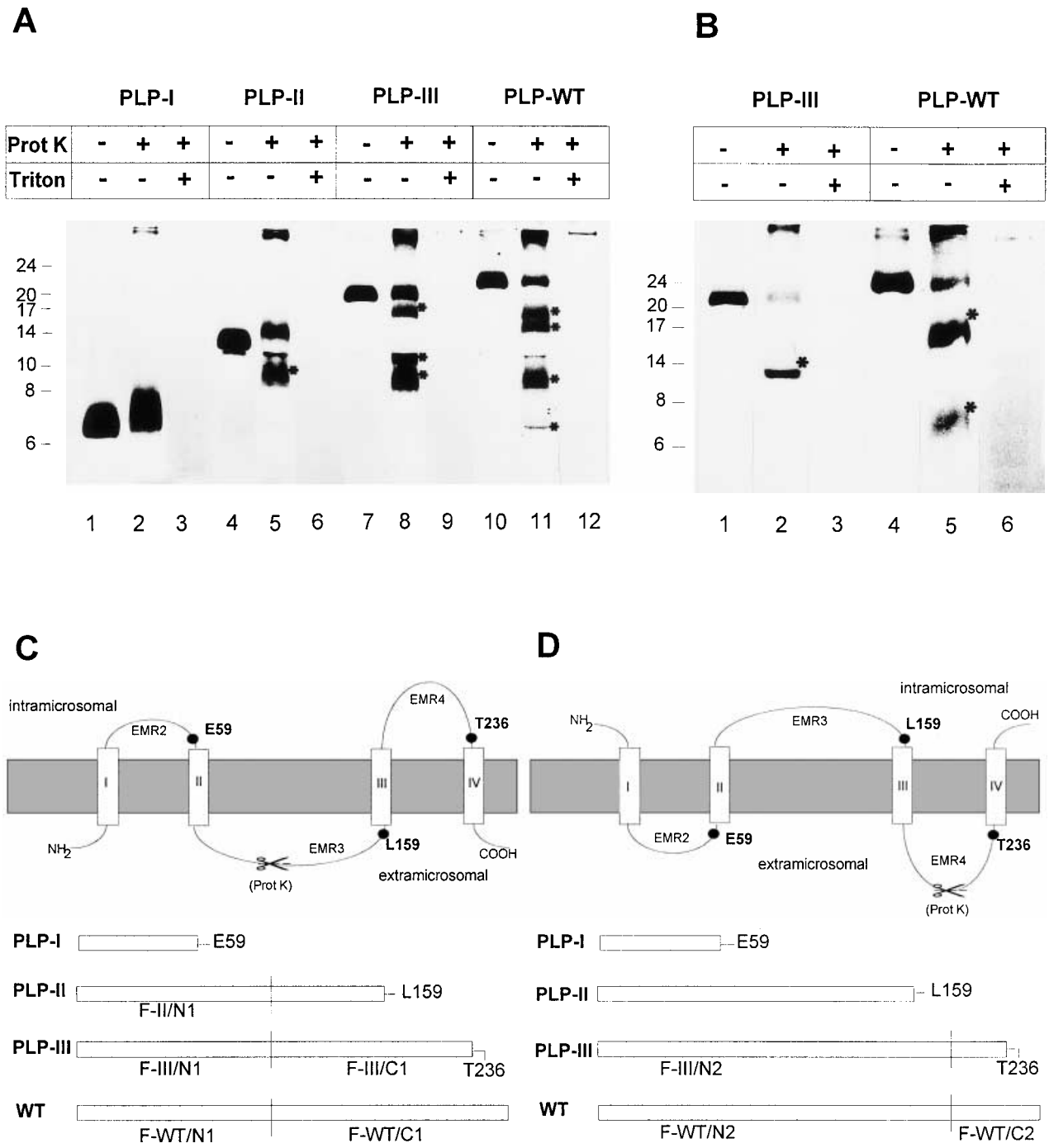

Fig. 3. Protein domain protection assays of radioactively labeled wild-type and truncated PLP polypeptides integrated into microsomal membranes. In vitro translation, proteinase K digestion, and sample analysis (16.5% Schägger gel) was performed as described in Materials and Methods. Proteolytic fragments are indicated by asterisks. **A:** Labeling with [^{3}H]leucine; 6.5 kDa PLP-I containing the hydrophobic TMDI domain was not degraded (lane 2); PLP-II truncated at position L159 yielded a 10 kDa fragment (F-II/N1) (lane 5). PLP-III truncated at T236 and wild-type PLP yielded a 19–20 kDa (F-III/N2, F-WT/N2) and 10 kDa fragment (F-III/N1, F-WT/N1). In addition, the shorter construct releases a 12–13 kDa (F-III/C1) and wild-type PLP a 16–17 kDa (F-WT/C1) and 6.5 kDa fragment (F-WT/C2), respectively (lanes 8 and 11). Protease treatment in the presence of Triton X-100 completely degraded the in vitro translated polypeptides. **B:** Labeling with [^{35}S]methionine. PLP-III with the C terminus at T236 yielded a 12–13 kDa segment (F-III/C1) (lane 2), whereas wild-type showed two segments with molecular masses of 16–17 kDa (F-WT/C1) and 6.5 kDa (F-WT/C2) (lane 5), respectively. Protease treatment in the presence of Triton X-100 completely degraded the in vitro translated polypeptides. **C,D:** Schematic drawing of PLP integrated in two different topologies into the microsomal membranes and representation of proteolytic fragments derived from protein domain protection assays of wild-type and truncated PLPs. Positions of stop codons introduced by site directed mutagenesis are indicated by black dots. C: Insertion of wild-type and truncated PLP into the membrane following the 4 TMH-N_{in} topology exposes the hydrophilic region EMR3 connecting TMDII and TMDIII at the extramicrosomal membrane surface. Proteolytic cleavage within this domain released F-II/N1 from PLP-II, F-III/N1 and F-III/C1 from PLP-III, and F-WT/N1 and F-WT/C1 from wild-type PLP. This agrees with the fragment pattern presented in Figure 3A,B. D: A 4-TMH-N_{out} topology would make two hydrophilic regions accessible for the protease at the extramicrosomal (cytoplasmic) membrane surface. No cleavage occurred in EMR 2 for reasons outlined in the Discussion. Single cleavage in EMR4 yielded F-III/N2 from PLP-III, and F-WT/N2 and F-WT/C2 from wild type.

of the respective extramembrane domains. N-glycosylation of consensus sequences occurs only in the lumen of the endoplasmic reticulum. N-glycosylation of the mutants P-N46I and P-N193S with a single consensus site monitored the luminal orientation of the mutagenized residues. Upon protein trafficking and sorting these tags representing EMR2 and EMR4 will be exposed at the outer surface of the plasma membrane. N-glycosylation of N113, however, challenged the cytoplasmic orientation of EMR3 proposed by the 4 TMH-N_{in}- model (Weimbs and Stoffel, 1992). Interestingly, each [^{35}S]methionine labeled PLP tagged with one N-glycosylation consensus sequence, when translocated into canine pancreas microsomal membranes, yielded roughly equal amounts of unmodified and monoglycosylated polypeptides (see Fig. 1). The lower molecular weight polypeptide represents the in vitro synthesized unglycosylated polypeptide integrated into the ER membrane presumably with an inversely oriented topology. A peripheral attachment to the microsomal membrane has been excluded. At least one consensus signal of the double mutants P-N46I/N113S and P-N113S/N193S and of the triple mutant P-N46I/N113S/N193S was posttranslationally modified by glycosylation. Only negligible amounts of unglycosylated translation products were visible. This indicated a) that the three consensus sites were obviously accessible for the N-glycosylation enzymes, and b) that the synthesis of oligosaccharide chains linked to the precursor proteins proceeds in the lumen of the vesicles nearly quantitatively. This is in agreement with reports on the in vitro expression of a variety of proteins, e.g., glucose transporter Glut1 (Hresko et al., 1994); glutamate transporter GLAST-1 (Wahle and Stoffel, 1996). N-glycosylation would fail if the glycosylation sites in the respective hydrophilic domains are located at the cytosolic surface or if the size, close proximity, or interaction with the luminal surface of the ER membrane prohibits the access for the glycosyltransferases (e.g., Singh et al., 1993; Wahle and Stoffel, 1996).

The glycosylation pattern of the different PLP constructs provided evidence for a position of the domains with asparagine residues N46 and N193 on one side and N113 on the other side of the ER membrane, whether on the inner or outer surface cannot be decided on this basis. Luminal orientation of N46/N193 and extramicrosomal of N113 would support a model with an extracellular orientation of EMR2 and EMR4 and an intracellular localization of EMR3 in the myelin membrane. The other topological orientation would correlate with the inverse orientation in a 4-helix N_{out} model (Popot et al., 1991).

From a similar study in vitro using mono N-glycosylation mutants (Gow et al., 1997), the conclusion was drawn that only the 4 TMH-N_{in}-topology of PLP occurs in the microsomal membrane. The discussion of these results did not refer to the significant amounts of unglycosylated products found in mutant PLPs with the consensus sites at position N46 of EMR2 and N221 in EMR4. Furthermore the N-glycosylation sequences introduced into EMR3 at N90 and N272 of EMR5 remained unmodified. These residues are located within the minimal distance of 12–14 amino acid residues between potential glycosylation site and luminal membrane surface required for efficient N-glycosylation (Nilsson and von-Heijne, 1993).

The second experimental approach to map the topology of PLP in the ER-membrane consisted in probing the protection of protein domains of PLP exposed on the luminal side and spanning the ER membrane against proteolytic degradation. The analytical data confirmed that the integration of PLP into the ER membranes follows two topological profiles. The pattern of proteolytic fragments of wild-type and truncated PLP indicated the cytosolic orientation of EMR3 in accordance with the 4 TMH-N_{in} and of EMR4 with the 4 TMH-N_{out} topology. Polypeptides with ipsilateral EMR2 and EMR4 oriented toward the outer (cytoplasmic) membrane surface did not release any proteolytic fragments derived from cleavage in both of these hydrophilic domains. One explanation might be the close proximity of the hydrophilic loop EMR2 to the outer membrane surface which prohibits the access of proteinase K. Previous studies suggested epitopes of PLP buried in the plasma membrane of intact oligodendrocytes and not accessible for polyclonal antibodies directed against the amino acid segment E43–E58 (Hudson et al., 1989). A recent report however describes the labeling of this domain by monoclonal antibodies recognizing PLP peptide 40-59 (Greer et al., 1996).

Another explanation for the missing proteolytic cleavage at these sites might be the reduced protease activity at the reaction temperature of 0°C required in the assay insufficient to cleave the short, approximately 20 residues long, hydrophilic domain. Even the larger domains EMR3 and EMR4 with 50-60 amino acids were only partially hydrolyzed at this temperature as indicated by the size of the proteolytic fragments. Incubation temperature at 10–20°C increased the enzyme activity but also led to permeabilization of the ER vesicle membrane and consequently complete degradation of the inserted polypeptides (data not shown).

Protein assembly in two alternative modes described here for PLP in the ER-membrane is quite uncommon but has also been reported for the hamster P-glycoprotein (Zhang et al., 1991, 1993). The question must be raised whether this is an artifact of the in vitro system, or does it reflect an intermediate stage in the ER-membrane of PLP expressing cells?

The in vitro system is functionally fully active in ribosome bound protein synthesis and contains the complete translocation machinery [Sec61p complex, TRAM protein, and signal recognition particle (SRP) receptor]. Even luminal factors like GRP78/BiP and GRP94 are present shown to be required to complete protein translocation (Nicchitta and Blobel, 1993).

In heterologously PLP expressing fibroplasts, PLP and BiP colocalize in the endoplasmic reticulum as demonstrated by immunofluorescence (Gow and Lazarini, 1996; Gow et al., 1994; Sinoway et al., 1994). The

heterologous in vitro system used in these studies resembles that of the nonglial cells. We conclude from our results that PLP is assembled in the ER membrane in two topologies, which correlate with the 4 TMH-N_{in} and -N_{out} profiles in the plasma membrane. Previous immunocytochemical studies have shown that PLP is present in the ER of transfected Cos-7 or HeLa cells but also moves through the secretory pathway to the plasma membrane (Gow et al., 1994; Sinoway et al., 1994). This has been documented by epitope mapping with monoclonal antibodies that recognize only sequences of EMR2 and EMR4 of PLP in the plasma membrane, which is integrated in the 4 TMH-N_{in} topology (Gow et al., 1997). Immunostaining revealed that PLP accumulates in large vesicles in the perinuclear region of many transfected cell lines and that PLP is transported to lysosomes for degradation (Gow et al., 1994). It is conceivable that only PLP in the appropriate 4 TMH-N_{in} conformation in the ER membrane can be sorted to the cell surface by vesicular transport, whereas inversely oriented PLP with N_{out} topology would be retained and degraded. The question whether N_{out}-PLP is also synthesized in myelinating glial cells has not yet been answered.

However oligodendrocytes might direct synthesis and ER-membrane integration of PLP exclusively in the 4 TMH-N_{in} topology by another mechanism. PLP has four unusually long putative transmembrane domains consisting of 30 and more predominantly hydrophobic amino acids. The length of these TMDs differs significantly from other polytopic proteins normally spanning lipid bilayer of only 20–30 Å width. Their most stable integration in-α helical conformation requires the complementary 50 Å wide lipid bilayer of myelin. Oligodendrocytes synthesize specific glycolipids such as cerebrosides and sulfatides, the ceramide part of which contains very long chain C_{18}-C_{26} fatty acids for the assembly of the hydrophobic core of the bilayer. Myelinating oligodendrocytes synthesize PLP and myelin specific lipids in the ER-membrane simultaneously (Schulte et al., 1993). This glycolipid environment might provide the suitable hydrophobic environment for the protein translocation channel for the polytopic integration of the four extended hydrophobic sequences of PLP.

In summary, we have used the in vitro system for the study of the biosynthesis, insertion, and the topology of PLP in the ER membrane. This system excludes "downstream" processes encountered in whole cell systems. We have shown that in the in vitro system PLP is integrated into canine pancreas ER-membranes in two topologies. The N_{in}-topology is in accordance with that of the PLP in myelin, the plasma membrane of oligodendrocytes and of heterologously PLP expressing cells.

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