

Neurons Promote the Translocation of Peripheral Myelin Protein 22 into Myelin

Sangeeta Pareek,¹ Lucia Notterpek,² G. Jackson Snipes,¹ Roland Naef,³ Wayne Sossin,¹ Jacynthe Laliberté,¹ Sandra Iacampo,¹ Ueli Suter,³ Eric M. Shooter,² and Richard A. Murphy¹

¹Montreal Neurological Institute and the Faculty of Medicine, McGill University, Montreal, Quebec H3A 2B4, Canada, ²Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305, and ³Institute of Cell Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Schwann cells express low levels of myelin proteins in the absence of neurons. When Schwann cells and neurons are cultured together the production of myelin proteins is elevated, and myelin is formed. For peripheral myelin protein 22 (PMP22), the exact amount of protein produced is critical, because peripheral neuropathies result from its underexpression or overexpression. In this study we examined the effect of neurons on Schwann cell PMP22 production in culture and in peripheral nerve using metabolic labeling and pulse-chase studies as well as immunocytochemistry. Most of the newly synthesized PMP22 in Schwann cells is rapidly degraded in the endoplasmic

reticulum. Only a small proportion of the total PMP22 acquires complex glycosylation and accumulates in the Golgi compartment. This material is translocated to the Schwann cell membrane in detectable amounts only when axonal contact and myelination occur. Myelination does not, however, alter the rapid turnover of PMP22 in Schwann cells. PMP22 may therefore be a unique myelin protein in that axonal contact promotes its insertion into the Schwann cell membrane and myelin without altering its rapid turnover rate within the cell.

Key words: Schwann cells; PMP22; glycosylation; turnover; Golgi; axonal contact

Peripheral myelin protein 22 (PMP22) is a component of compact peripheral nerve myelin that is produced by Schwann cells (SC) (Suter et al., 1993; Snipes and Suter, 1995). PMP22 appears to be essential for peripheral nerve function, as determined by genetic studies. Mutations in the mouse *pmp22* gene have been found in Trembler (*Tr*) (Suter et al., 1992a) and Trembler-J (*Tr^J*) mice (Suter et al., 1992b), both of which have severely demyelinated peripheral nerves. Point mutations in the human *PMP22* gene have also been identified in some Charcot-Marie-Tooth disease type 1A (CMT1A) families (Valentijn et al., 1992b; Roa et al., 1993a,b) and in the clinically more severe hypertrophic neuropathy Dejerine–Sottas syndrome (Roa et al., 1993c). However, in most CMT1A patients the *PMP22* gene is duplicated (Matsunami et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Valentijn et al., 1992a). Furthermore, a heterozygous deletion of the same chromosomal region that is duplicated in CMT1A families has been detected in patients with hereditary neuropathy with liability to pressure palsies (HNPP) (Chance et al., 1993, 1994).

A CMT1A transgenic rat model has been generated that over-

expresses PMP22. These animals develop SC hypertrophy, muscle weakness, and reduced conduction velocities (Sereda et al., 1996) similar to CMT1A patients. Moreover, PMP22-deficient transgenic mice show delayed onset of myelination, tomacula-like focal myelin thickenings followed by severe demyelination, axonal loss, and functional impairment similar to what is seen in HNPP (Adlkofer et al., 1995). All these findings emphasize that the absolute level of PMP22 protein is of critical importance for correct myelin formation and maintenance and, in turn, focus on how the synthesis and targeting of PMP22 in SC in nerve is regulated.

Similar to PMP22, the expression level and proper processing of other myelin proteins is of critical importance for normal glial cell biology (Scherer and Chance, 1995). For example, heterozygous and homozygous deletions of the protein zero (P0) gene lead to demyelinating and dysmyelinating phenotypes, respectively (Martini et al., 1995). Furthermore, point mutations in P0 are associated with a heterogeneous group of dysmyelinating disorders (Warner et al., 1996). One possible mechanism for some of the observed differences in clinical phenotypes is that each specific mutation uniquely alters the trafficking of the mutated P0 protein. Recent studies in our laboratories show that there is a similar functional heterogeneity with respect to *pmp22* mutations present in the *Tr* and *Tr^J* mice. In *Tr*, PMP22 is retained in the endoplasmic reticulum (ER) (Naef et al., 1997), whereas in *Tr^J*, PMP22 accumulates in lysosomal compartments of the Schwann cells (Notterpek et al., 1997).

As a step toward understanding the pathogenesis of the observed neuropathies, one must define the normal trafficking of the wild-type PMP22 protein. Like the other peripheral myelin proteins, PMP22 mRNA and protein synthesis is significantly up-regulated in SC by axonal contact (Snipes et al., 1992; Bosse et al., 1994; Suter et al., 1994). In exploring this aspect of PMP22

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S.P. and L.N. contributed equally to this work.

Correspondence should be addressed to Dr. Eric M. Shooter, Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305.

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biology further, we have previously shown that, unlike the major myelin protein P0, PMP22 has a short half-life in SC in culture (Pareek et al., 1993). We now show that this characteristic feature of PMP22 synthesis does not change in SC that are in contact with axons in co-culture or in nerve. Moreover, unlike P0, PMP22 is only targeted to the SC membrane in detectable amounts when SC myelinate axons.

MATERIALS AND METHODS

Tissue culture. SC cultures were prepared from neonatal rat sciatic nerves according to the methods of Brookes et al. (1979) and modified as described previously (Pareek et al., 1993). SC were maintained in medium supplemented with 5 μ M forskolin to stimulate myelin-related gene expression (Lemke and Chao, 1988; Pareek et al., 1993).

Dissociated cultures of sensory neurons from Sprague Dawley rat embryos were established as described previously (Kleitman et al., 1991). Briefly, embryonic day 15 dorsal root ganglia (DRG) were enzymatically and mechanically dissociated. Cells were plated on 12 mm collagen-coated glass coverslips for morphological studies or on collagen-coated tissue culture plastic for biochemical analysis. The day after the dissection, the cultures were treated with fluorodeoxyuridine (10 μ M) for at least three cycles to remove non-neuronal cells.

Co-cultures of DRG neurons and SC (DRG-SC) were established as described by Einheber et al. (1993). Briefly, SC were harvested by trypsin treatment (0.05% containing 0.02% EDTA) and plated onto established DRG neurons. SC were allowed to proliferate and attach to neurons. Myelin synthesis was induced by incubating the cultures in medium containing 15% fetal calf serum, 50 μ g/ml ascorbic acid, and 20 ng/ml progesterone (Koenig et al., 1995). Cultures were maintained for up to 3 months. Myelin production was confirmed by immunocytochemical and biochemical methods.

Immunocytochemistry. SC and co-cultures were fixed in 4.0% paraformaldehyde in PBS for 10 min at room temperature followed by postfixation and permeabilization in 100% methanol for 10 min at -20° C. Samples were blocked by incubation in 20% normal goat serum containing PBS for at least 30 min at room temperature. Primary antibodies were added in the same blocking solution for 2–3 hr at room temperature or overnight at 4° C (co-cultures). A previously characterized rabbit polyclonal antibody (Corinne) raised against synthetic peptide of the rat PMP22 (amino acids 117–132) (Notterpek et al., 1997) was used at dilution of 1:250 for staining of the co-cultures. A second polyclonal anti-PMP22 antibody (Andy) raised against the corresponding human peptide was used for immunostaining of the SC. In this region, sequences of the rat and human PMP22 are 81% homologous, and antibodies to the two species cross-react. For double-immunolabeling studies, a mouse monoclonal antibody to P0 (a gift from Dr. J. J. Archelos, Bayerische Julius-Maximilians Universität, Würzburg, Germany), antibody to neurofilament (NF) (Dako, Carpinteria, CA), and an antibody to 58 kDa Golgi protein (Sigma, St. Louis, MO) were used. After three 10 min rinses in PBS, we added fluorochrome-conjugated secondary antibodies from goat, including FITC-conjugated anti-mouse IgG and IgM (Boehringer Mannheim, Indianapolis, IN), and Texas Red-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for 1–2 hr at room temperature. Preimmune and peptide-adsorbed rabbit antisera were used as controls. Control samples without primary antibodies were processed in parallel. Coverslips were mounted with Citifluor (University of Kent, Canterbury, UK), and samples were photographed using a Nikon Microphot FXA microscope.

Metabolic labeling. Cells in culture and sciatic nerve explants were metabolically labeled with 0.4 mCi/ml *trans*- 35 S label (1100 Ci/mmol; ICN Biochemicals, Costa Mesa, CA) as described (Pareek et al., 1993). For pulse-chase studies, cells were incubated in *trans*- 35 S label containing medium for 30–45 min, washed twice with prewarmed DMEM, and incubated in fresh medium with an excess of cold methionine and cysteine for varying time intervals.

Immunoprecipitation. SC and DRG-SC co-cultures were solubilized on ice for 45 min in 1.5 ml of precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% deoxycholate, 0.5% Nonidet P-40, and 0.1% SDS) containing 20 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 20 μ g/ml leupeptin. Cell lysates were immunoprecipitated with a previously characterized polyclonal antibody raised against rat PMP22 peptide (amino acids 117–132) (Pareek et al., 1993) or with antibodies to P0 (a gift from Dr. Bruce Trapp, Cleveland Clinic Foundation, Cleveland,

OH) as described previously (Pareek et al., 1993). For endoglycosidase H (Endo H) treatment, agarose beads containing complexes of radiolabeled antigen and antibody were resuspended in 200 μ l of 20 mM sodium phosphate, pH 6.0, and 20 mM NaCl. For *N*-glycosidase F (PNGase F) treatment, agarose beads were resuspended in 200 μ l of 200 mM sodium phosphate buffer, pH 7.6, containing 0.1% SDS and 10 mM β -mercaptoethanol. After 5 min incubation, a nonionic detergent Nonidet P-40 was added to a sevenfold excess over SDS, before the addition of PNGase F. One-half of each sample received Endo H (5 mU; Boehringer Mannheim) or PNGase F (1.25 U; Genzyme, Cambridge, MA), and the other half received buffer without enzyme. Samples were incubated for 16–18 hr at 4° or 37° C for Endo H and at 37° C for PNGase F and then washed three times in (in mM) 2 EDTA, 0.5 dithiothreitol, and 10 Tris-HCl, pH 7.5, at 4° C. The beads were resuspended in SDS sample buffer (in %: 2 SDS, 5 β -mercaptoethanol, 10 glycerol, and 0.001 bromophenol blue), boiled for 5 min, and analyzed by SDS-PAGE using a 13–22% gradient gel system. After electrophoresis, gels were fixed in 40% methanol and 10% acetic acid and treated with ENHANCE (DuPont NEN, Boston, MA) according to the manufacturer's instructions. Dried gels were exposed to Kodak XAR film and developed on an X-Omat film processor. Densitometric analyses of the autoradiographs were performed using National Institutes of Health Image software. Identical methods were used to analyze homogenates of metabolically labeled sciatic nerve explants.

Western blot analysis. Purified rat myelin membranes were prepared from adult rat sciatic nerves by the method of DeVries et al. (1978). Endoglycosidase digestions, PNGase F and Endo H (both from New England Biolabs, Beverly, MA) were performed according to the manufacturer's suggestions. Sciatic nerve homogenates, purified myelin, lysates of DRG-SC co-cultures, and SC alone were prepared directly in the denaturation buffer supplied with the enzymes. Protein concentrations were determined by the method of Lowry et al. (1951). As a positive control for Endo H activity, 2 μ g of RNaseB (Sigma) was added to the SC lysates. Samples with and without enzyme were incubated for 16 hr at 37° C. Proteins were separated on 12.5% SDS gels under reducing conditions, and gels were transferred to nitrocellulose membranes (Towbin et al., 1979). After blocking with 5% skim milk in PBS, blots were incubated with the same anti-rat PMP22 antiserum (Corinne; 1:1000) that was used for the co-culture immunocytochemical studies in 5% goat serum containing PBS for 16 hr at 4° C. Bound antibodies were detected using the ECL detection method (Amersham, Arlington Heights, IL). Cleavage of RNaseB by Endo H and PNGase F was monitored by Coomassie blue staining of parallel gels and by Ponceau S staining of the nitrocellulose membranes before Western blotting.

RESULTS

Most newly synthesized PMP22 in cultured SC remains in the ER

We have previously shown that PMP22 is expressed in SC in a glycosylated form that is generated from an 18 kDa precursor, and that the turnover rate of PMP22 in these cells is rapid, with a half-life of 30–60 min (Pareek et al., 1993). To determine where within the cell the degradation of newly synthesized PMP22 occurs, we metabolically labeled SC, immunoprecipitated PMP22, and treated the precipitate with Endo H before electrophoresis (Fig. 1A). Endo H removes high mannose-containing, noncomplex sugar residues from newly synthesized proteins. Because glycoproteins become Endo H resistant only after cleavage by mannosidase II, which is located within the medial Golgi compartment (Kornfeld and Kornfeld, 1985), Endo H sensitive glycoproteins are thought to be located within the ER. Endo H resistant, complex carbohydrate-containing proteins are found in the Golgi compartment. Figure 1A shows that most of the newly synthesized PMP22 is sensitive to Endo H treatment, indicating its location within the ER, and is rapidly degraded within 30–60 min. Only a few percent of the newly synthesized, labeled PMP22 remains after a 60 min chase (Fig. 1A). Most of this protein (78%) has a slightly elevated molecular mass (Fig. 1A, right arrow) and is resistant to Endo H.

To test whether our culture conditions were artificially promot-

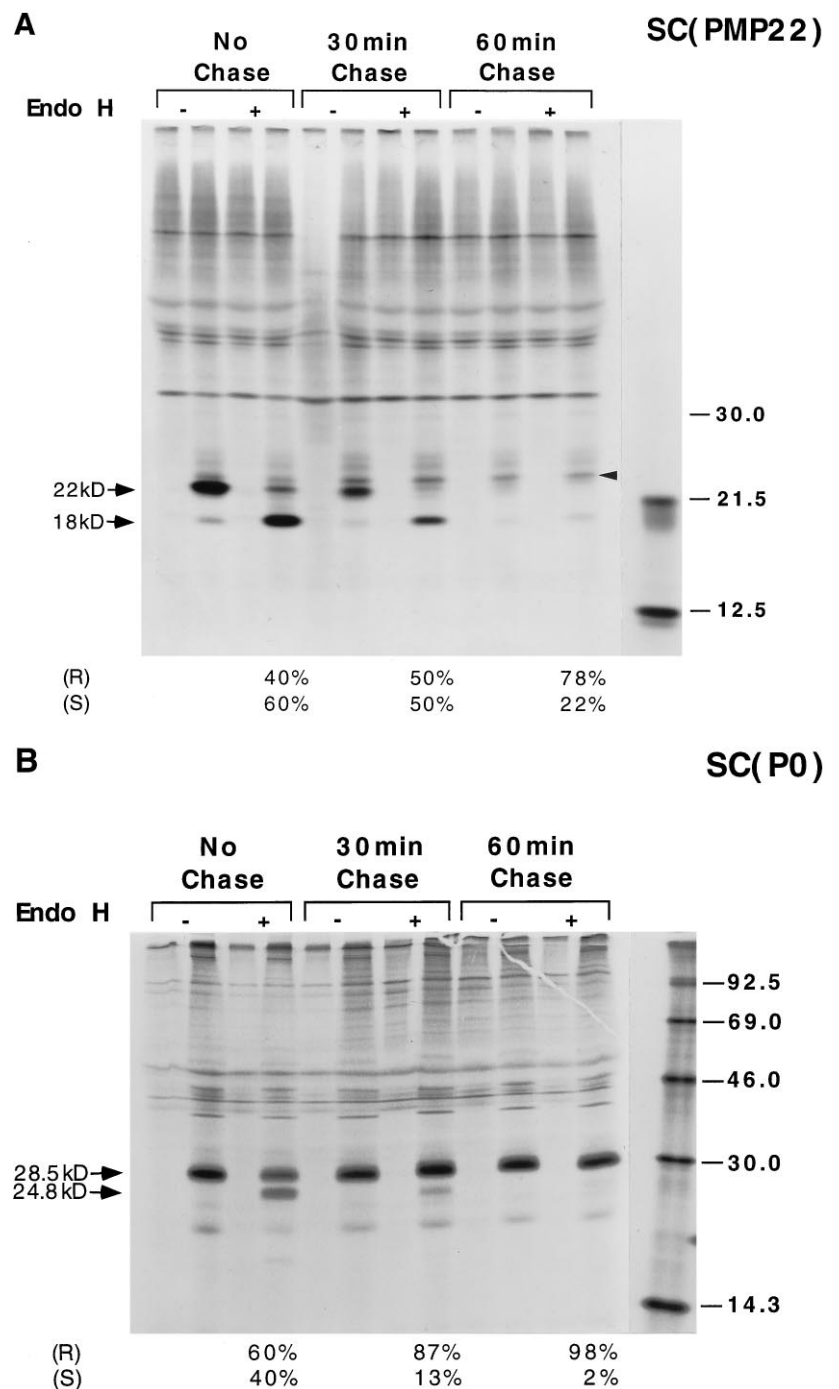


Figure 1. Pulse-chase analysis and Endo H digestion of PMP22 (*A*) and P0 (*B*) in SC. SC were metabolically labeled with *trans*-³⁵S label for 30 min and chased for 30 and 60 min with cold methionine and cysteine. SC lysates were immunoprecipitated with either PMP22 (*A*) or P0 (*B*) antibodies, and the immunoprecipitates were incubated with (+) or without (-) Endo H at 4°C for 16–18 hr. After immunoprecipitation, cell lysates were analyzed by SDS-PAGE and fluorography. Duplicate samples were precipitated with preimmune serum (*first lane* in each group). The migration positions of PMP22 and P0 are indicated by arrows. *Top arrows* show the glycosylated proteins, and *bottom arrows* show the unglycosylated proteins. The *right arrow* in *A* shows the Endo H resistant PMP22. Molecular weight markers are shown at the extreme *right* in both *A* and *B*. Endo H resistant (*R*) and sensitive (*S*) PMP22 (*A*) and P0 (*B*) are shown as the percentage of total protein at different time intervals.

ing the rapid disappearance of PMP22, we monitored in identical samples the turnover rate of P0, the major SC-derived glycoprotein of peripheral nerve myelin. In contrast to PMP22, almost all of the newly synthesized P0 remained after a 60 min chase period (Fig. 1*B*). Furthermore, within 30 min of chase, most of the newly synthesized P0 (87%) was converted to an Endo H resistant form. Therefore, under these culture conditions, SC transfer P0 from the ER to the Golgi compartment and process the carbohydrate moiety of the protein.

A complex glycosylated form of PMP22 accumulates over time

Although it represents only a small fraction of the protein produced in SC, the Endo H resistant form of PMP22 accumulates

over time. Figure 2 compares SC cultures that were metabolically labeled either for a short (45 min) or long term (6 or 16 hr). After immunoprecipitation, the samples were incubated in the presence or absence of Endo H or PNGase F. In cells radiolabeled for 6 or 16 hr, the labeled PMP22 is more heterogeneous than PMP22 labeled for 45 min (Fig. 2, *top arrow*). That all these bands contribute to PMP22 was confirmed by digestion with PNGase F, which completely removes the carbohydrate moiety from PMP22. This digestion changed the molecular mass of all these bands downward by ~4 kDa, the difference between the glycosylated and unglycosylated forms of PMP22 (Fig. 2, *bottom arrow*). Whereas ~70% of the short-term-labeled (45 min) PMP22 is Endo H sensitive, only ~20% of the long-term-labeled PMP22 is

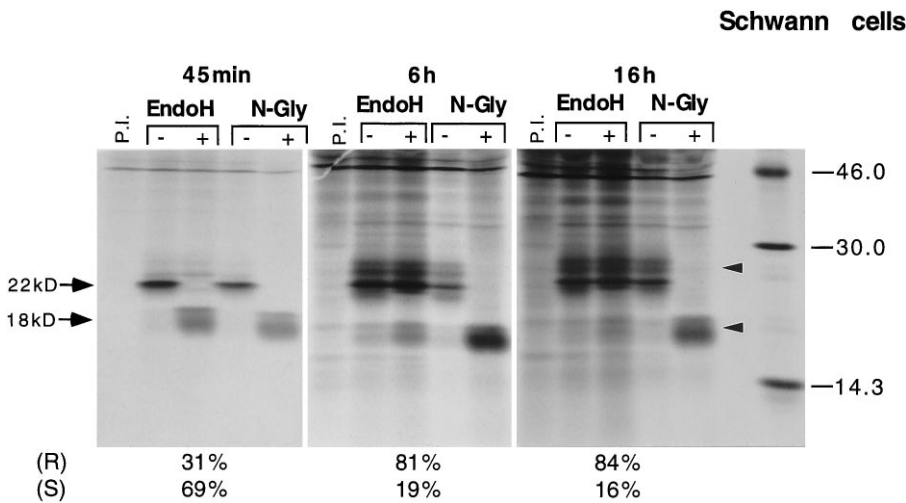


Figure 2. Comparison of short- and long-term labeling of cultured SC. SC were metabolically labeled with *trans*-³⁵S label for 45 min, 6 hr, or 16 hr. Cell lysates were immunoprecipitated with PMP22 antibodies, and the immunoprecipitates were incubated with (+) or without (-) Endo H or PNGase F (*N-Gly*) at 37°C for 16–18 hr. After immunoprecipitation, cell lysates were analyzed by SDS-PAGE and fluorography. Duplicate samples were treated with preimmune serum (*first lane* in each group). The migration positions of glycosylated 22 kDa PMP22 (*top arrow*) and the 18 kDa core peptide (*bottom arrow*) are indicated. The *top right arrow* shows the Endo H resistant accumulated PMP22 protein, and the *bottom right arrow* shows the 18 kDa core protein that appears after PNGase F digestion. Molecular weight markers are shown at the extreme *right*. Endo H resistant (*R*) and sensitive (*S*) PMP22 are shown as the percentage of total protein at different time intervals.

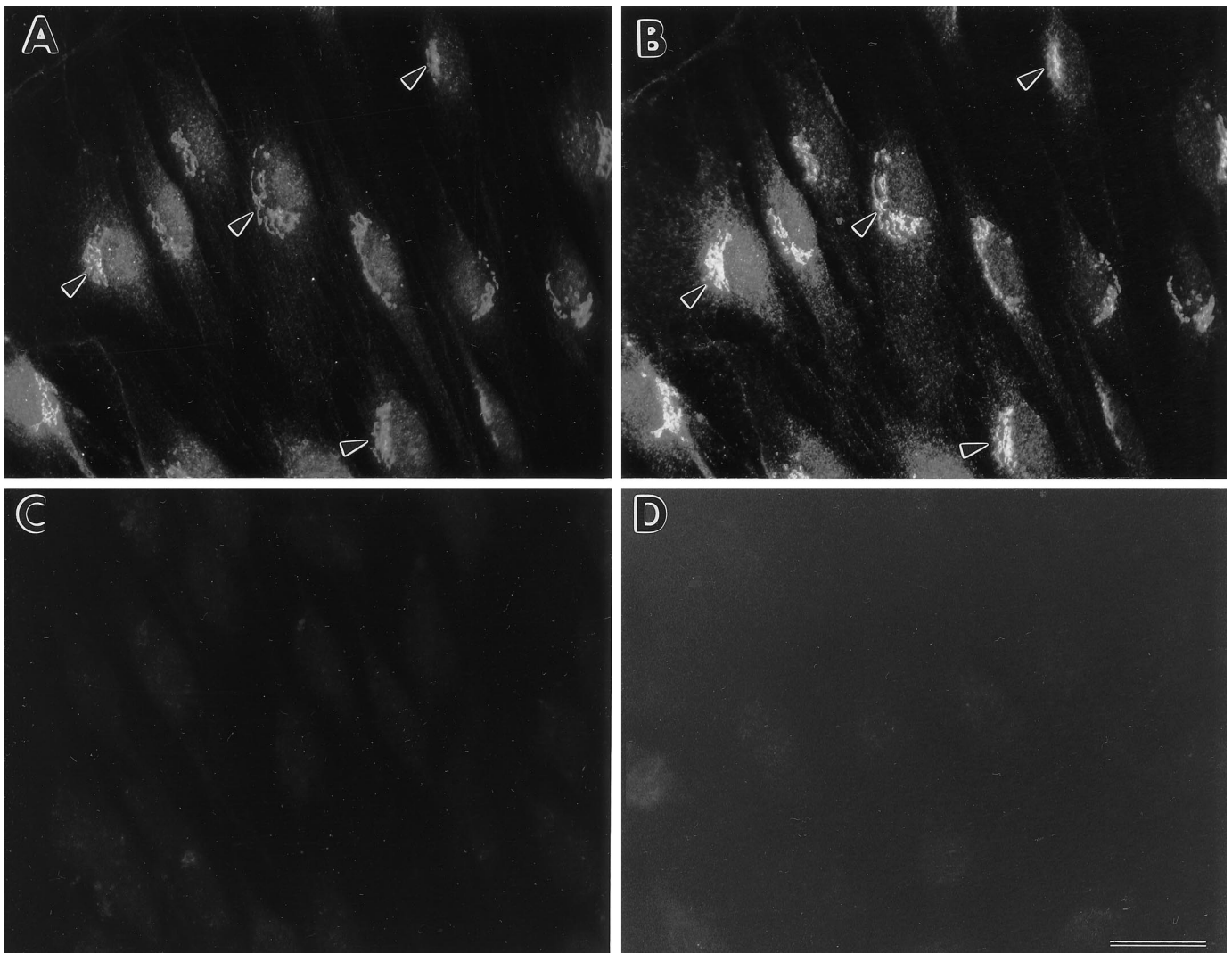


Figure 3. Immunocytochemical localization of PMP22 in cultured SC. SC cultures were fixed in 4.0% paraformaldehyde and permeabilized by treatment with methanol. Samples were double-stained with PMP22 antisera visualized by Texas Red-conjugated anti-rabbit IgG and monoclonal Golgi marker (FITC). The distribution of PMP22 (*B*) and the 58 kDa Golgi protein (*A*) are shown. Most of the PMP22-like immunoreactivity co-localized with the Golgi marker (*arrows* in *A, B*). Controls are shown in which the samples were incubated with PMP22 peptide-adsorbed antiserum (*C*) or preimmune serum (*D*). Scale bar (shown in *D*), 22 μm.

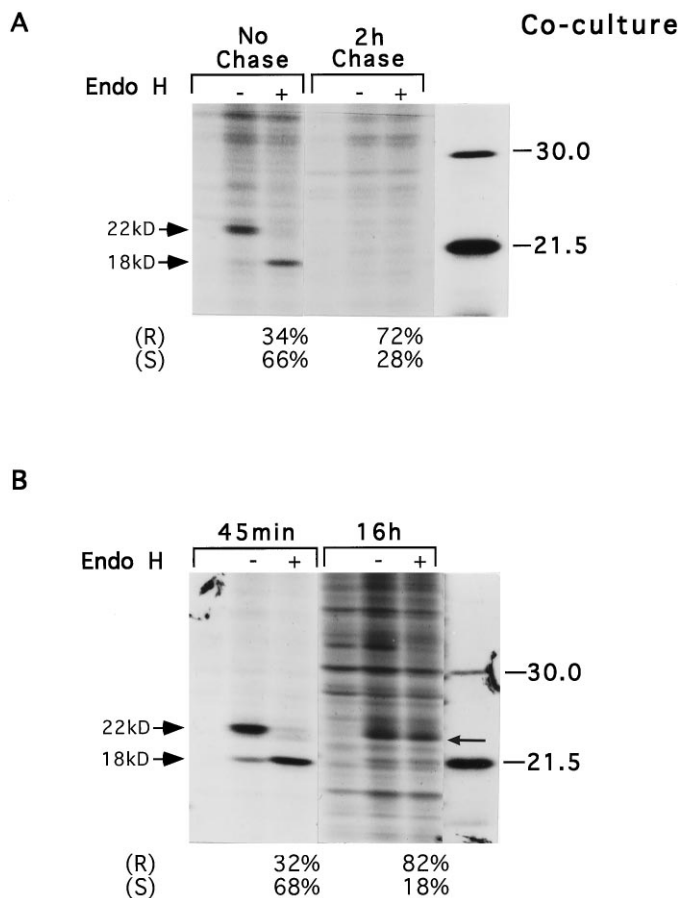


Figure 4. Pulse-chase analysis (*A*) and short- and long-term labeling (*B*) of PMP22 in myelinating co-cultures of SC and neurons. DRG-SC co-cultures were grown in the presence of ascorbic acid and progesterone. Cells were metabolically labeled with *trans*-³⁵S label for 45 min and chased for 2 hr with cold methionine and cysteine (*A*). For comparison of short- and long-term labeling studies (*B*), myelinating co-cultures were metabolically labeled with *trans*-³⁵S label for either 45 min or 16 hr. Cell lysates were immunoprecipitated with PMP22 antibodies, and the immunoprecipitates were incubated with (+) or without (–) Endo H at 4°C for 16–18 hr. After immunoprecipitation, the lysates were analyzed by SDS-PAGE and fluorography. Duplicate samples were precipitated with pre-immune serum (*first lane* in each group). The migration positions of glycosylated PMP22 (*top arrows*) and the 18 kDa core peptide (*bottom arrows*) are indicated. The *right arrow* in *B* indicates the accumulated Endo H resistant PMP22 protein. Molecular weight markers are shown at the extreme *right*. Endo H resistant (*R*) and sensitive (*S*) PMP22 are shown as the percentage of total protein at different time intervals.

Endo H sensitive. Therefore, the PMP22 that accumulates within SC over time must be the small proportion of newly made stable, Endo H resistant protein.

Stable PMP22 immunoreactivity is detectable within the Golgi compartment of SC

Although the rapidly degraded PMP22 is located within the ER, most of the stable Endo H resistant PMP22 accumulates in the Golgi compartment. This was evident in colocalization immunocytochemical analysis using polyclonal antibodies to PMP22 (Fig. 3*B*) and a monoclonal Golgi marker (Fig. 3*A*). The majority of the PMP22 immunoreactivity, representative of the steady-state levels of PMP22 in rat SC, was found in the Golgi compartment. A second anti-PMP22 antiserum against the same rat peptide sequence showed low levels of PMP22 distributed more broadly

over the cytoplasm, but still with marked concentration of PMP22 in the Golgi (data not shown). These differences may be attributable to the two antibodies recognizing alternative forms or epitopes of PMP22 as noted earlier (Pareek et al., 1993). PMP22 immunoreactivity was never seen in the cell membrane of non-myelinating SC in culture either before or after permeabilizing the cells.

Biosynthesis of PMP22 in co-cultures of SC and neurons

To determine whether axonal contact could alter the dynamics of PMP22 production or its localization within SC, we studied PMP22 biosynthesis in DRG-SC co-cultures under conditions that promote myelination. Co-cultures were grown for 7 d, and then myelination was induced by adding ascorbic acid and progesterone to the medium. Three weeks later, the cultures were metabolically labeled for 45 min and chased for 2 hr, and cell extracts were immunoprecipitated with antibodies to PMP22. Aliquots of the samples were also treated with Endo H before electrophoresis. After this short-term radiolabeling period, most of the PMP22 (~70%) synthesized by SC cultured with neurons is Endo H sensitive (Fig. 4*A*). Moreover, after a 2 hr chase only trace amounts of labeled PMP22 remained (Fig. 4*A*). The rapid turnover of PMP22 in the ER, characteristic of SC alone, is therefore also observed in myelinating SC in culture. In cultures radiolabeled for 16 hr, 82% of the accumulated PMP22 is Endo H resistant (Fig. 4*B*, *right arrow*), a result similar to that obtained in SC grown in the absence of neurons (Fig. 2). The accumulation of Endo H resistant PMP22 in myelinating SC occurs with about the same time course as it does in SC alone.

Immunocytochemical localization of PMP22 in co-cultures of SC and neurons

DRG-SC co-cultures were also examined immunocytochemically to monitor the steady-state distribution of PMP22. In 1-week-old co-cultures grown in medium without ascorbate and progesterone, PMP22 staining was localized to the perinuclear region of SC (Fig. 5*B*, *arrows*). This distribution is similar to that of PMP22 in cultures of nonmyelinating SC (Fig. 3*B*), although the accumulation in the Golgi compartment is not so obvious. Double immunolabeling with the anti-NF antibody (Fig. 5*A*, *arrows*) showed that the SC are in contact with axons. After an additional 4 weeks in medium containing ascorbate and progesterone (when myelination had occurred), PMP22 was still seen in the SC, but a significant fraction appeared in myelin (Fig. 5*D*, *arrows*) along neuro-filament-positive neuronal processes (data not shown). The distribution of PMP22 in myelin (Fig. 5*D*) was similar to that of P0 (Fig. 5*C*). Contact with axons therefore promotes the transport of PMP22 from the Golgi compartment to myelin.

Ex vivo labeling of rat sciatic nerve confirms the rapid degradation of newly synthesized PMP22

To determine how closely the biosynthesis and transport of PMP22 in DRG-SC co-cultures mimicked that in peripheral nerve *in vivo*, we used explants of 10-d-old rat sciatic nerves. The explants were metabolically labeled for 30 min and then chased for different time intervals. Parallel samples were treated with Endo H to determine the intracellular localization of PMP22. The results showed that most of the newly synthesized PMP22 (65%) is sensitive to Endo H digestion, indicating that the protein is located in the ER and contains high mannose rather than complex carbohydrates (Fig. 6). In these respects the biosynthesis of PMP22 in whole nerve is like that in SC in culture alone or

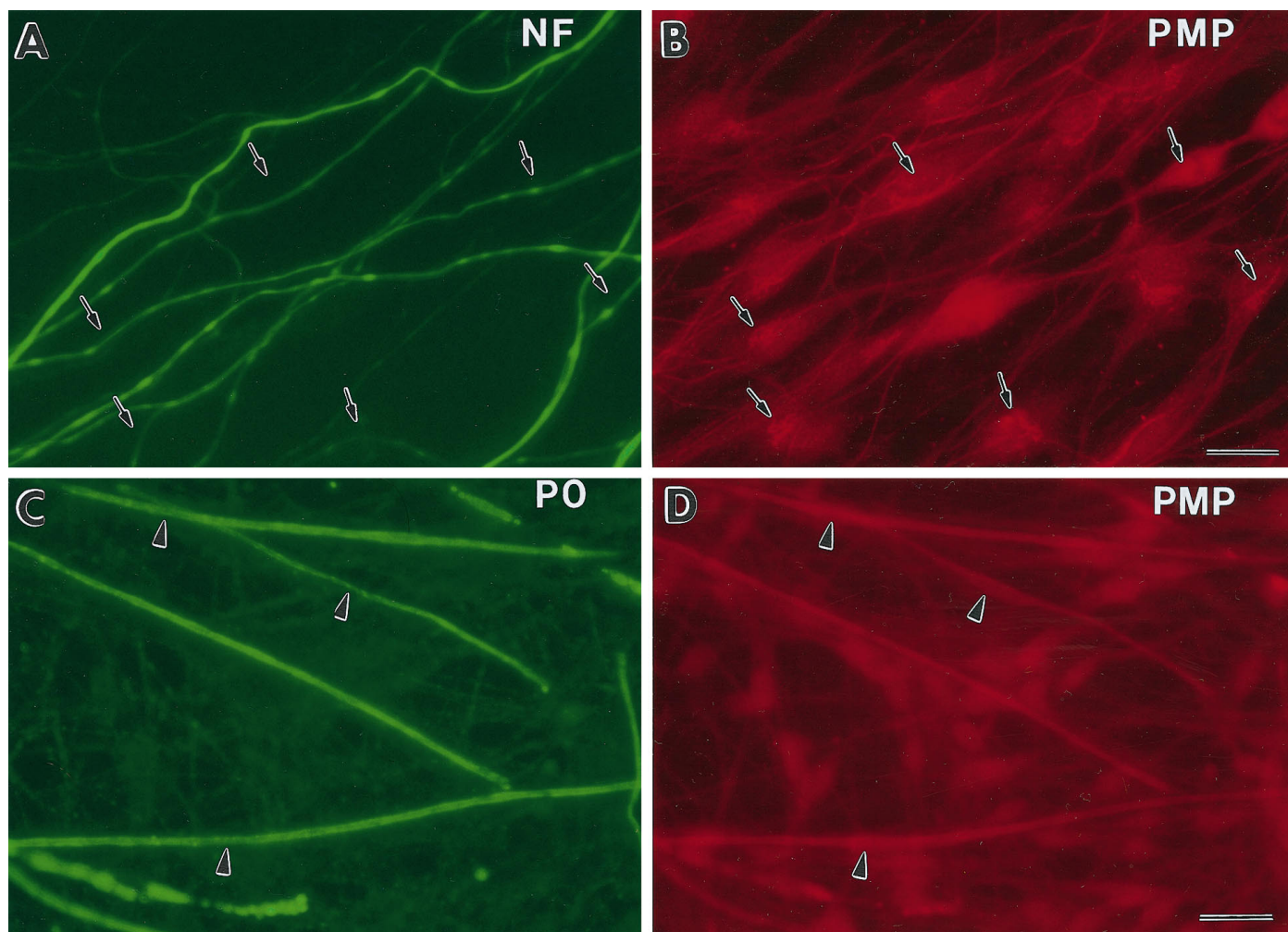


Figure 5. Immunolocalization of PMP22 in short- and long-term myelinating co-cultures. One-week-old SC and neuron co-cultures were double-stained with monoclonal anti-NF (*A*) and polyclonal PMP22 antiserum (*B*). Arrows point to SC (*B*) that are in contact with neuronal processes (*A*). After 4 weeks in medium that promotes myelination, PMP22 (*D*) co-localizes with P0 reactive myelin segments (*C*). Arrows point to the cell bodies of elongated SC (*D*) with uniform PMP22 staining over the cell membrane. Scale bars: *A*, *B* (shown in *B*), 22 μm ; *C*, *D* (shown in *D*), 25 μm .

with neurons. Also, the protein is rapidly degraded (Fig. 6), with a turnover time approximating that observed in SC alone (Fig. 1*A*) or DRG-SC co-cultures (Fig. 4*A*). This rapid turnover of newly synthesized PMP22 in sciatic nerve occurs in the presence of easily detectable levels of stable PMP22 (by Western analysis) in the nerve myelin throughout the chase period (data not shown). A difference between sciatic nerve explants and SC alone or co-cultures is that the process of conversion of Endo H sensitive to Endo H resistant PMP22 is much slower in sciatic nerve explants. After a 60 min chase period, >70% of total PMP22 becomes Endo H resistant in SC and co-cultures whereas in sciatic nerve explants only 40% of total PMP22 is Endo H resistant. This difference may be caused by the slow down of sciatic nerve metabolism in our *ex vivo* conditions.

PMP22 is complex-glycosylated in nonmyelinating and myelinating cells under steady-state conditions

The data from long-term metabolic labeling suggested that PMP22 becomes Endo H resistant because it accumulates in the Golgi compartment or myelin. To confirm this, the steady-state PMP22 protein in nonmyelinating SC, 8-week-old myelinating co-cultures, 10-d-old sciatic nerve, and purified myelin from adult nerve was analyzed for sensitivity to Endo H. Under all condi-

tions studied PMP22 was largely Endo H resistant (Fig. 7). Interestingly, low levels of Endo H sensitive PMP22 were also observed under myelinating conditions. All the Endo H resistant forms of PMP22 were reduced to the same 18 kDa core protein when treated with PNGase F, an enzyme that removes the single carbohydrate moiety of PMP22 (Fig. 7). The differences in mobility of the glycosylated PMP22 in SC compared with SC under myelinating conditions (Fig. 7) are, therefore, attributable to variations in the composition of the PMP22 carbohydrate side chains. Similar differences in the migration pattern were also seen for newly accumulated PMP22 in SC alone (Fig. 2) and in co-cultures (Fig. 4*B*).

DISCUSSION

Metabolic labeling experiments show that most of the newly synthesized PMP22 produced by SC in culture is rapidly turned over and is sensitive to the action of Endo H, suggesting localization to the ER. A small portion of the PMP22 produced during this labeling period, however, becomes complex-glycosylated, as measured by resistance to Endo H. The same profiles were observed for PMP22 biosynthesis in myelinating co-cultures of SC and neurons and in sciatic nerve explants radiolabeled *ex vivo*.

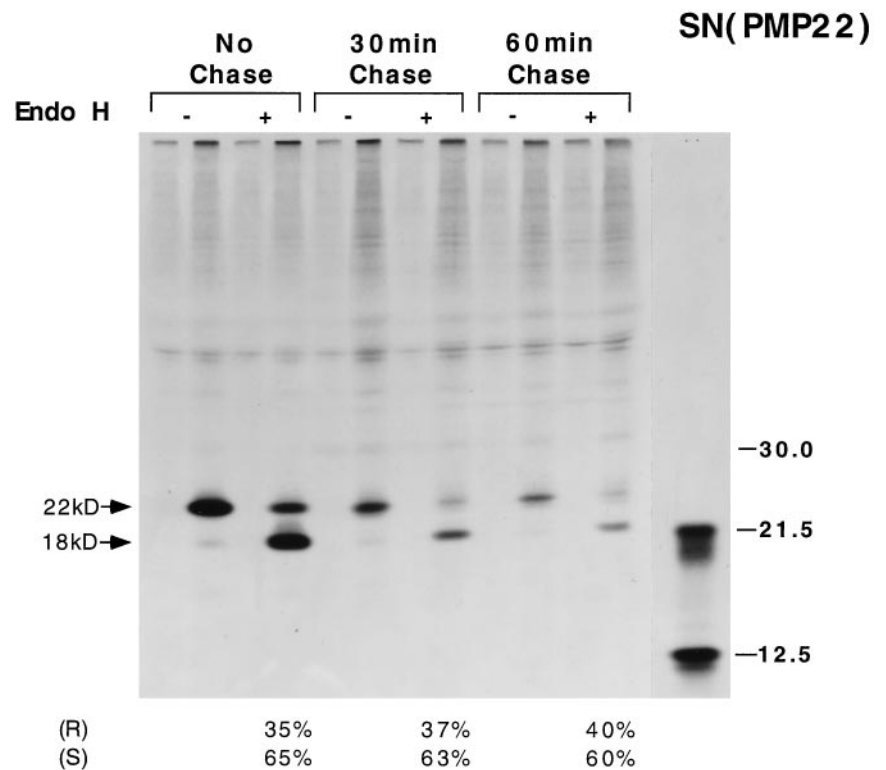
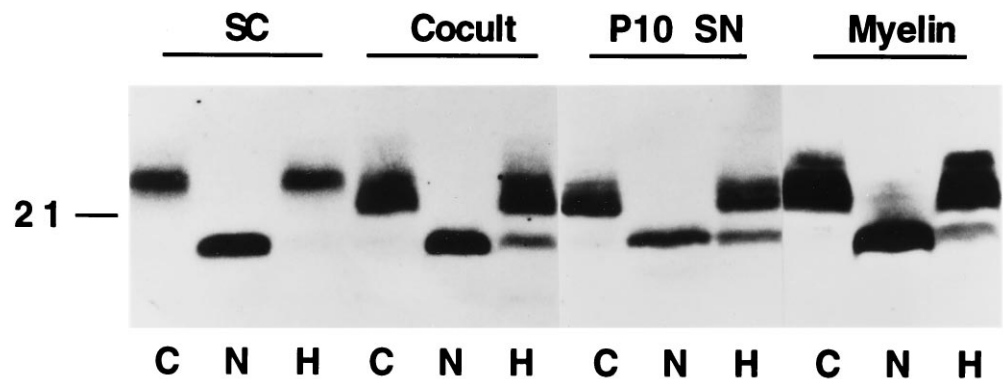


Figure 6. Pulse-chase analysis of *ex vivo*-labeled sciatic nerve explants. Ten-day-old sciatic nerve segments were metabolically labeled with *trans*-³⁵S label for 30 min and chased for 30 and 60 min with cold methionine and cysteine. Nerve homogenates were immunoprecipitated with PMP22 antibodies, and the immunoprecipitates were incubated with (+) or without (–) Endo H at 4°C for 16–18 hr. After immunoprecipitation, nerve samples were analyzed by SDS-PAGE and fluorography. Duplicate samples were precipitated using preimmune serum (*first lane* in each group). The migration positions of glycosylated 22 kDa PMP22 (*top arrow*) and the 18 kDa core peptide (*bottom arrow*) are indicated. Molecular weight markers are shown at the extreme *right*. Endo H resistant (*R*) and sensitive (*S*) PMP22 are shown as the percentage of total protein at different time intervals.

Figure 7. Western blot analysis of PMP22 in *in vitro* and *in vivo* samples. PMP22 in nonmyelinating SC (50 μg/lane total protein), 8-week-old myelinating co-cultures (50 μg/lane), 10-d-old sciatic nerve (10 μg/lane), and purified myelin (10 μg/lane) was tested for the presence of high-mannose and complex carbohydrates by PNGase F (*N*) and Endo H (*H*) treatments as described in Materials and Methods. Control samples incubated without the addition of enzymes are analyzed in *lanes C*. Molecular masses are indicated in kilodaltons.



The process of myelination does not appear to alter significantly the initial steps in PMP22 biosynthesis in SC. Almost 70–80% of newly synthesized PMP22 is rapidly degraded in the ER in all conditions studied. Long-term metabolic labeling of PMP22 in SC indicates that the small fraction of Endo H resistant PMP22 seen after short-term labeling increases to become the predominant form of PMP22. Again, the same picture emerges in myelinating SC in culture and in nerve. PMP22 in peripheral nerve myelin is also largely Endo H resistant. Taken together, these results indicate that stable, predominantly Endo H resistant forms of PMP22 arise as a result of the processing and accumulation of only a small fraction of the total PMP22 produced by the SC, a process that is not altered significantly during myelination.

What does change during myelination is the intracellular localization of the stable Endo H resistant PMP22. In SC alone PMP22 accumulates in the Golgi compartment. When axons are present and myelination occurs, PMP22 moves into the myelin membrane. Under identical experimental conditions the dynamics of PMP22 biosynthesis are significantly different from those of P0, the major SC myelin glycoprotein. In agreement with earlier

studies (Poduslo, 1984; Poduslo et al., 1985), we found that most of the P0 produced by SC in culture acquires complex glycosylation and Endo H resistance shortly after its synthesis, and the protein remains stable during the 1 hr chase period. These properties are consistent with the rapid transit of P0 to the SC membrane even in SC cultured alone (L. Notterpek, unpublished data). P0, unlike PMP22, does not require a neuronal signal to become membrane-associated. Furthermore, studies by Brunden and Poduslo (1987) and Brunden et al. (1990a,b) suggest that SC use a post Golgi compartment for the degradation of P0. In the absence of myelin assembly, in transected nerve and in SC in culture, most of the newly synthesized P0 is degraded within a 3 hr time period via the lysosomal pathway.

The finding that most of the PMP22 produced by SC is Endo H sensitive and rapidly turned over suggests that the cell uses some mechanism for degrading PMP22 within the ER. ER degradation of newly synthesized proteins occurs through a nonlysosomal pathway and requires specific signals. For type I transmembrane proteins such as the α -chain of the T-cell receptor, charged residues in the transmembrane domain are important determi-

nants of ER degradation (Bonifacino et al., 1991). In type II transmembrane proteins, including the H2a subunit of the asialoglycoprotein receptor, sequences in the juxtamembrane region specifically target the protein to the ER degradative pathway (Lederkremer and Lodish, 1991; Wikström and Lodish, 1992; Yuk and Lodish, 1993). PMP22 has four putative transmembrane domains, but none contains charged amino acid residues. Rat PMP22 does have a short amino acid sequence (Asn-Gly-His-Arg-Thr) immediately C-terminal to the first membrane-spanning domain that resembles the sequence that targets the rapid degradation within the ER of the H2a subunit of the asialoglycoprotein receptor (Glu-Gly-His-Arg-Gly) (Lederkremer and Lodish, 1991). This sequence may have a similar function with respect to the rapid degradation of PMP22 in the ER.

The significance of accumulation of PMP22 in the Golgi compartment is unknown. It may relate to a yet unidentified role for PMP22 that differs from its function in myelin. PMP22 is a ubiquitous protein (Manfioletti et al., 1990; De Leon et al., 1994; Parmantier et al., 1995), and fibroblasts, among other cells, also produce and rapidly turn over PMP22 (S. Pareek and R. A. Murphy, unpublished data). In SC, retention of PMP22 within the Golgi compartment could be necessary for its targeting to the cell membrane. The “kin recognition” model (Nilsson et al., 1994) suggests that homodimeric proteins may form large oligomers in the Golgi network that prevent their inclusion into transport vesicles. We have observed multimerization of PMP22 *ex vivo* under nondenaturing conditions (data not shown). PMP22 may also require transient interactions with chaperone proteins or cell-specific PMP22-binding proteins to initiate transfer from the ER and Golgi compartments to the plasma membrane. These chaperones may be absent from nonmyelinating SC.

Although both Endo H sensitive and resistant PMP22 were reduced to the 18 kDa core protein by PNGase F treatment, some heterogeneity was evident in the migration pattern of the glycosylated 22 kDa protein. These size differences, which probably result from variations in the carbohydrate content of the protein, could be significant in altering the functional properties of the molecule. PMP22 contains the L2/HNK-1 adhesion and recognition carbohydrate epitope (Snipes et al., 1993) that may be important during myelinogenesis and in the maintenance of compact myelin. In nonmyelinating and myelinating SC, steady-state PMP22 contains predominantly complex (Endo H resistant) oligosaccharides; however, in myelinating cells a detectable level of Endo H sensitive PMP22 is also present. Similar results have been obtained for P0 (Brunden, 1992). In 5-d-old neonatal animals, most of the P0 is complex glycosylated and Endo H resistant, although in the adult, only 60% of the protein contains complex sugars (Brunden, 1992).

These studies show that most newly synthesized PMP22 is rapidly turned over in the ER. However, a small amount of complex glycosylated PMP22 is accumulated in the Golgi, which is targeted to the membrane under myelinating conditions. Mutations in PMP22 disturb the PMP22 trafficking in the SC in unique ways. The effect of the L16P mutation in PMP22 in *Tr^r* mice does not appear to alter the PMP22 targeting pathway up to its insertion in myelin; however, the myelin that is formed is unstable and undergoes autophagy and/or endocytosis in the endosomal–lysosomal pathway (Notterpek et al., 1997). In contrast, the G160D mutation in *Tr* mice prevents the movement of the mutated PMP22 to myelin, and the protein is accumulated in the ER (Naef et al., 1997). It will be of great interest to determine how overexpression or underexpression of PMP22, characteristic

of the human peripheral neuropathies, disturbs a system in which much of the normal PMP22 is already degraded in the ER. Animal models for such studies are now available.

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