Peripheral myelin protein, 22 kDa (PMP22), is a myelin molecule associated with Schwann cells in peripheral nerves (Snipes, G. J., Suter, U., Welcher, A. A., and Shooter, E. M. (1992) J. Cell Biol. 117, 225–238). Mutations affecting the PMP22 gene have been implicated in the trembler mutation in mice (Suter, U., Welcher, A. A., Ozelik, T., Snipes, G. J., Kosaras, B., Francke, U., Billings-Gagliardi, S., Sidman, R. L., and Shooter, E. M. (1992) Nature 356, 241–244; Suter, U., Moskow, J. J., Welcher, A. A., Snipes, G. J., Kosaras, B., Sidman, R. L., Buchberg, A. M., and Shooter, E. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4382–4386) and Charcot-Marie-Tooth Disease in humans (Patel, P. L., Roa, B. B., Welcher, A. A., Schoener-Scott, R., Trask, B. J., Pentao, L., Snipes, G. J., Garcia, C. A., Francke, U., Shooter, E. M., Lupsiki, J. R., and Suter, U. (1992) Nature genet. 1, 159–165). In this report, we have studied PMP22 production in cultured rat Schwann cells. Schwann cells contain a 1.8-kilobase mRNA transcript coding for PMP22, and its production is up-regulated in vitro by forskolin. Metabolic labeling combined with immunoprecipitation methods using antibodies raised against synthetic peptides of PMP22 reveal that Schwann cells generate the protein from an 18-kDa precursor form which is post-translationally modified by N-linked glycosylation. A second molecule (molecular mass, 48 kDa) that reacted with PMP22 antibodies was also detected in Schwann cells but is not related chemically to PMP22 as determined by pulse-chase labeling. Metabolic labeling of rat sciatic nerve and Western blot analyses of purified rat sciatic nerve myelin reveal that deglycosylation of PMP22 gives rise to an 18-kDa protein similar in size to that in Schwann cells. These results indicate that cultured Schwann cells may provide a good model in which to investigate the production and function of PMP22 and to establish the cellular basis for the protein's involvement in inherited peripheral neuropathies.

Peripheral nerve myelin is formed by the ensheathment and compaction of Schwann cell processes around competent axons (Morell et al., 1989). Several major structural myelin proteins have been identified in compact myelin, including protein zero (P₀), myelin basic protein (MBP), and myelin-associated glycoprotein. Recently, an additional member of this list, termed peripheral myelin protein, 22 kDa (PMP22), has been identified (Welcher et al., 1991; Snipes et al., 1992). The protein is associated structurally with Schwann cell membranes and, like other proteins of compact myelin, is produced when myelin is formed (Snipes et al., 1992). Levels of PMP22 mRNA and protein in peripheral nerves decline when myelin is disrupted as a result of nerve crush injuries and increase when axons regenerate (De Leon et al., 1991; Welcher et al., 1991; Spreyer et al., 1991; Snipes et al., 1992).

The biological role of PMP22 is not known but the protein appears to play an essential role in peripheral nerve function. The trembler (Tr) mouse mutant carries an autosomal dominant point mutation in the PMP22 gene (Suter et al., 1992a) and a second point mutation affecting PMP22 has been found in the allelic trembler-J (Tr/J) mouse (Suter et al., 1992b). Tr and Tr/J mice show limb paralysis, tremor, transient seizures, as well as severe peripheral nervous system-specific hypomyelination and continuous Schwann cell proliferation. In humans, the gene coding for PMP22 has been directly implicated in the most common form (1 in 2,500 individuals; Skre, 1974) of inherited human peripheral neuropathy, Charcot-Marie-Tooth disease type 1A (CMT1A; Patel et al., 1992; Valentijn et al., 1992; Timmerman et al., 1992; Matsunami et al., 1999). The majority of CMT1A cases are associated with a chromosomal duplication involving ∼1.5 megabases on the short arm of human chromosome 17 (Lupsaki et al., 1991; Raeymaekers et al., 1991; Hoegendijk et al., 1991). The PMP22 gene maps to the CMT1A duplication region, but is not interrupted by the CMT1A duplication.
(Patel et al., 1992), suggesting that a gene dosage effect involving PMP22 might be responsible for the demyelinating neuropathy that occurs in CMT1A patients (Patel et al., 1992). Understanding how these two putative mutant mechanisms, point mutations in Tr and Tr' and gene duplication in CMT1A, lead to peripheral nervous system myelin deficiencies will require an understanding of the biological function(s) of PMP22. As a first step toward this goal, we have sought to establish an appropriate in vitro system in which we can study the biology of PMP22.

In this study, we demonstrate that PMP22 is produced by cultured Schwann cells and that its production, like that of other myelin proteins, can be regulated by forskolin which elevates intracellular CAMP concentrations. Using metabolic labeling and immunoprecipitation methods, we have determined that cultured Schwann cells produce PMP22 in an N-linked glycosylated form that arises from an 18-kDa precursor which can also be detected in sciatic nerve. In the course of these studies, we also detected in cultured Schwann cells, but not in sciatic nerve, a second protein (molecular mass, 46 kDa) that is distinct from but immunologically related to PMP22 in that it can be precipitated with an antipeptide antibody. Our data suggest that cultured Schwann cells may be a useful model for studying the regulation and biological functions of PMP22, information that will be essential for understanding its role in peripheral nerve disease.

MATERIALS AND METHODS

Tissue Culture—Schwann cell cultures were prepared from neonatal rat sciatic nerve according to the methods of Brockes et al. (1979), as modified previously (Barker et al., 1991), except that nerve fragments were dissociated in 0.25% trypsin, 0.03% collagenase, and cultures were exposed to two cycles of cysteine arabinoside (10 μm) treatment each day to eliminate fibroblasts. Purified Schwann cells were grown on poly-L-lysine (100 μg/ml; Sigma)-coated tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), gentamicin (50 μg/ml), glycerol, and surface probability and secondary structure predictions to be essential for understanding its role in peripheral nerve disease.

Immunoprecipitation—Schwann cells were solubilized on ice for 45 min in 1 ml of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM diethiothreitol) followed by low salt (2 mM EDTA, 0.5 mM diethiothreitol, 10 mM Tris-HCl, pH 7.5) containing buffer at 4 °C. Washed beads were suspended in 200 μl of 10 mM Tris (pH 7.5), 1% Triton X-100, 0.5% deoxycholate, 0.5% Tween 20, 20 mg/ml heat-denatured salmon sperm DNA, 10% glycerol, and 0.001% bromophenol blue) and centrifuged for 2 min prior to electrophoresis. Identical methods were used for analyzing homogenates of sciatic nerves.

Electrophoresis—Samples from metabolic labeling studies were analyzed by 12%–22% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were fixed in 40% methanol and 10% acetic acid and treated with ENHANCE (Du Pont-New England Nuclear) according to the manufacturers instructions. Dried gels were exposed to Kodak XAR film at −80 °C and developed on an X-omat film processor.

Northern blot Analysis—Total RNA was isolated from freshly collected Schwann cell cultures and neonatal rat sciatic nerves using the guanidine hydrochloride method (Chomczynski and Sacchi, 1987). Total RNA (2.5 μg) was completely degraded with approximately 10°C of 32P-labeled cDNA probe in 30 μl of hybridization buffer containing 80% formamide, 40 mM PIPES, pH 6.4, 0.4 μM NaCl, and 1 mM EDTA, heated to 95 °C for 15 min, and transferred to a 55 °C water bath for 18 h. Samples were supplemented with 350 μl of RNAse digestion buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 5 mM EDTA) containing 40 μg/ml RNase A (Boehringer Mannheim) and 1200 units/ml RNAse T1 (Bethesda Research Laboratories) and incubated for 1 h at 37°C. The samples were treated with RNase P, 0.1% SDS, 1% deoxycholate, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM diethiothreitol) followed by low salt (2 mM EDTA, 0.5 mM diethiothreitol, 10 mM Tris-HCl, pH 7.5) containing buffer at 4 °C. Washed beads were suspended in 200 μl of 10 mM Tris (pH 7.5), 1% Triton X-100, 0.5% deoxycholate, 0.5% Tween 20, 20 mg/ml heat-denatured salmon sperm DNA, 10% glycerol, and 0.001% bromophenol blue) and centrifuged for 2 min prior to electrophoresis. Identical methods were used for analyzing homogenates of sciatic nerves.

Northern blot Analysis—RNA was fractionated by electrophoresis through 1.2% agarose/formaldehyde gels and transferred overnight to support nitrocellulose transfer membrane (Schleicher & Schuell) in 10 × SSC (sodium citrate, 1 × SSC contains 0.15 M NaCl, 0.015 M Na3Citrate, pH 7.0) and baked at 80 °C prior to the addition of 24-26° C hybridization buffer containing 0.2 μCi/ml [32P]-labeled cDNA probe. Hybridized filters were washed twice with preheated formamide, 5 × SSC, 5 × Denhardt's solution (0.5% Ficoll, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin), 0.1% SDS, and 100 μg/ml heat-denatured salmon sperm DNA (10 μg/ml). The filters were washed twice with preheated formamide, 5 × SSC, 5 × Denhardt's solution (0.5% Ficoll, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin), 0.1% SDS, and 100 μg/ml heat-denatured salmon sperm DNA (10 μg/ml) and were exposed to X-Omat film.
filters were washed twice at room temperature (5 min each) in 2 × SSC, 0.1% SDS followed by one wash at 65 °C for 30 min in 2 × SSC, 0.1% SDS. Finally, the filters were washed twice at 65 °C (30 min each) in 0.2 × SSC, 0.1% SDS. Filters were air-dried, and autoradiography was performed as described (Sambrook et al., 1989).

**Hybridization Probes**—For RNase protection assays, a 356-base pair BamHI/HindII fragment containing nucleotides 143–499 of the rat PMP22 cDNA (Welcher et al., 1991) was subcloned into the pGEM-3 vector (Promega), and radiolabeled antisense cRNA probes were transcribed with SP6 polymerase (Bethesda Research Laboratories) and [32P]CTP (800 Ci/mmol; Du Pont-New England Nuclear) as described previously (Melton et al., 1984). Template DNA was removed with 5 units RNase-free DNase (Boehringer Mannheim) in the presence of 20 units RNAsin (Promega) in a total volume of 25 µL. Reaction mixtures were extracted once with pheol/chloroform and ethanol-precipitated. The probe was electrophoresed on a denaturing acrylamide gel (6% acrylamide, 8 M urea, 1 × TBE) and exposed to XAR film for 1 min; the full-length RNA band was identified and excised, using the film as a template, and eluted in 400 µL of elution buffer (2 M ammonium acetate, 1% SDS, 50 µg/mL yeast tRNA) for 2 h at 37 °C. RNA was ethanol-precipitated and resuspended in 100 µL of hybridization buffer. For Northern blot analysis, cDNA probes were labeled with [3ZP]dCTP to a specific activity of >109 cpm/µg of DNA using the random hexamer procedure (Feinberg and Vogelstein, 1984).

**Western Blot Analysis**—Myelin membranes were isolated by sucrose gradient ultracentrifugation from 6-week-old rats as described by Smith and Perret (1986) except that the 10.5% sucrose homogenization buffer contained 1 mM phenylmethylsulfonyl fluoride and 4 mM o-phenanthroline. Twenty micrograms of protein were applied to 12% polyacrylamide minigels, electrophoresed, and transferred to Immobilon membranes and detected as described (Snipes et al., 1992), except that 1% normal goat serum was added to all blocking and antibody dilution buffers. The membranes were probed with anti-peptide 2 antisera (1:2000) for 2 h followed by a peroxidase-conjugated secondary antibody. The product was visualized by peroxidase-catalyzed chemiluminescence (Amersham) according to the manufacturer's instructions.

**Endoglycosidase Treatment**—Twenty micrograms of myelin membrane protein were reacted with peptide-2-β-N-acetyl-β-glucosaminyl asparagine amidase (PNGase F, N-glycanase; Genzyme Corp.) overnight at 37 °C with minor modifications of the reaction conditions suggested by the manufacturer (50% of the suggested detergent concentrations (SDS and Nonidet P-40) and 50% of N-glycanase concentration, 0.15 units/20 µg of myelin protein). The reaction product was electrophoresed on a 15% SDS acrylamide minigel.

**RESULTS**

Detection of PMP22 in Cultured Schwann Cells in Vitro—Schwann cells grown in medium containing forskolin produce a 1.8-kilobase transcript coding for PMP22 (Fig. 1C) identical in size to that detected in sciatic nerve (Welcher et al., 1991; Snipes et al., 1992). Cultured Schwann cells produce PMP22 mRNA at considerably lower levels than neonatal rat sciatic nerves (5–10% as determined by densitometry), suggesting that in situ Schwann cells produce more PMP22 than in culture. Removal of forskolin from the culture medium results in a down-regulation of PMP22 mRNA (Fig. 1A, lane c) which is reversed when forskolin is added back to medium (Fig. 1A, lanes d and e). This result is in full agreement with previous studies, repeated here (Fig. 1B), showing that forskolin increases 5–10-fold Schwann cell production of mRNA coding for P0 (Lemke and Chao, 1988).

PMP22 protein is also produced by cultured Schwann cells and, like PMP22 mRNA, its synthesis is forskolin-regulated. Fig. 2 shows the results of studies in which we metabolically labeled Schwann cells with [35S]methionine and immunoprecipitated cell lysates with a mixture of antisera raised against two synthetic peptides from PMP22 (peptide 1, amino acids 27–42; peptide 2, amino acids 117–132) representing regions predicted to be exposed on the protein's surface (Fig. 3). In these experiments, a molecule (Fig. 2, middle arrow) was immunoprecipitated that electrophoresed on SDS-PAGE as a doublet with an apparent molecular mass of 22 kDa (Fig. 2, lanes b, d, and f). This doublet (PMP22) was not detected in lysates treated with antisera preincubated with excess amounts of peptides 1 and 2 (Fig. 2, lanes a, c, and e) or in samples immunoprecipitated with preimmune serum (data not shown). Only trace amounts of the protein were evident in Schwann cells grown 3 days in the absence of forskolin (Fig. 2, compare lanes b and d). PMP22 levels elevate when forskolin is added back to the culture medium (Fig. 2, lane f). Fig. 2 also shows that large amounts of a higher molecular weight protein (48 kDa, indicated by the upper arrow on the figure) were specifically immunoprecipitated with the anti-PMP22 antisera mixture. Unlike PMP22, however, levels of this 48-kDa molecule were unaffected by forskolin (Fig. 2, lanes b, d, and f).

Fig. 4 shows the results of metabolic labeling experiments in which we immunoprecipitated Schwann cell extracts with antisera to peptides 1 and 2 separately instead of a mixture of the two. Antisera to peptide 1 precipitated the 48-kDa molecule but not PMP22, whereas antisera to peptide 2 recognized PMP22 and not the 48-kDa protein. Both antisera also specifically immunoprecipitated small amounts of an 18-kDa molecule which is indicated by the lower arrow on the
Combined peptides. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The migration positions of 35S-labeled synthetic peptide. Amino acid numbering refers to the structure of the immunoprecipitation experiments (Figs. 2 and 4).

Schwann cells cultured in complete medium in the presence of [35S]methionine for 4 h and the cell lysates immunoprecipitated with a mixture of antisera to PMP22 peptides 1 and 2 in the presence (lanes a, c, and e) or absence (lanes b, d, and f) of the combined peptides. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The migration positions of 35S-labeled PMP22 and a co-precipitating 48-kDa protein are indicated by arrows.

Peptide 1  
\[^{27}\text{Gln-Trp-Leu-Gly-Asn-Gly-His-Arg-Thr-Asp-Leu-Trp-Gln-Asn-Cys}^{52}\text{COOH}\]

Peptide 2  
\[^{31}\text{Tyr-Thr-Val-Arg-Ser-Glu-Trp-His-Val-Asn-Asp-Tyr-Ser-Tyr}^{132}\text{-Cys-COOH}\]

This result suggests that PMP22 and the 48-kDa molecule are not chemically related and do not have a precursor-product relationship.

Immunoprecipitations testing the specificity of antibodies to peptide 1 and peptide 2 from PMP22. Cultured Schwann cells, metabolically labeled with [35S]methionine for 4 h, were solubilized in radioimmune precipitation buffer, and extracts were immunoprecipitated with antibodies to peptide 1 or peptide 2, separately or together, in the presence (bIP) or absence (IP) of blocking concentrations (1 mg/ml) of the respective peptides. The migration positions of 35S-labeled PMP22 and the unidentified 48-kDa protein are indicated by arrows. The figure shows that antibody to peptide 1 recognizes the 48-kDa protein but not PMP22, whereas antisera to peptide 2 recognizes PMP22 and not the 48-kDa protein.

To examine this possibility further and also to investigate the role of glycosylation in the generation of PMP22, we repeated the metabolic labeling studies using Schwann cells incubated in medium containing tunicamycin which inhibits N-linked glycosylation. Fig. 6 shows that tunicamycin-treated Schwann cells, immunoprecipitated with antibodies to peptide 1 (left panel) or peptide 2 (right panel), contain an 18-kDa protein but not the 22-kDa PMP22. This result suggests that Schwann cell-derived PMP22 is normally glycosylated. It should also be recalled that antibodies to peptide 1 do not recognize processed PMP22 (Fig. 4) but do immunoprecipitate small amounts of the 18-kDa protein from tunicamycin-treated cultured Schwann cells. Since peptide 1 represents an amino acid segment of PMP22 that contains a consensus site for N-linked glycosylation, it seems reasonable that antibody to peptide 1 may recognize PMP22 protein in the absence but not presence of N-linked carbohydrates in cultured Schwann cells.

Immunocytochemical Localization of PMP22 in Cultured Schwann Cells—We carried out immunocytochemical studies to determine the localization of PMP22 in cultured Schwann cells that are not elaborating myelin. Fixed Schwann cells were immunostained with the PMP22-specific antipeptide 2 antiserum in order to avoid the possible specificity problems of anti-PMP22 peptide 1 antiserum in vitro. Fig. 7 shows that antibodies to peptide 2 detect PMP22 bound to cultured cells.
Schwann cells with heaviest staining evident on cells flattened onto the culture substratum. Staining was distributed throughout the cytoplasm and was not specifically localized on the cell membrane which might be expected for a myelin-based protein. A similar staining pattern has been described for P0 in cultured Schwann cells (Morgan et al., 1991).

PMP22 Is a Myelin Glycoprotein with an 18-kDa Polyepitope Core in Vivo—We questioned whether the metabolic labeling and immunoprecipitation results we obtained with PMP22 in isolated Schwann cells would be similar or different from those obtained with sciatic nerve in vivo. Accordingly, we carried out short term metabolic labeling experiments followed by immunoprecipitations on sciatic nerve segments maintained for 3 h in tissue culture medium in the presence or absence of tunicamycin. Fig. 8 shows that in nerve samples labeled ex vivo in the absence of tunicamycin, antibodies to peptides 1 and 2 immunoprecipitated PMP22 which migrated on SDS-PAGE as a doublet with mobility identical to that of PMP22 in cultured Schwann cells. It should be recalled that antibody to peptide 1 did not recognize mature PMP22 in Schwann cell extracts (Fig. 4). In nerve segments incubated in medium containing tunicamycin, the 18-kDa protein was immunoprecipitated with both antibodies, as it was from extracts of cultured Schwann cells (compare Figs. 6 and 8). The antipeptide 1 antiserum precipitated the 18-kDa species more completely than the 22-kDa species in sciatic nerve ex vivo.

PMP22 was also analyzed in myelin purified from sciatic nerves of young adult rats. Fig. 9 is a Western blot replica of isolated sciatic nerve myelin that had been treated with recombinant N-glycanase which catalyzes the hydrolysis of asparagine-linked oligosaccharides at the asparagine residue; the blot was developed with antiserum to peptide 2. In the panel on the left (A) a molecule which reacts with PMP22 peptide antibodies (lane 2) and has a size of 22 kDa can be seen in the Coomassie-stained gel (lane 1) migrating between P0 and MBPs, both of which are identified on the figure. B shows that the mobility of the 22-kDa molecule following glycanase treatment is shifted to the 18-kDa form. These results are identical to those obtained with tunicamycin in metabolic labeling studies of isolated Schwann cells (Fig. 6) and sciatic nerve segments (Fig. 8) and suggest that PMP22 in sciatic nerve myelin, like that in cultured Schwann cells, has a core size of 18 kDa and is glycosylated to a form with an apparent molecular size of 22 kDa. Antiserum against peptide 1 gave the same results as antiserum against peptide 2, and no 48-kDa protein was detected by Western blot in sciatic nerve extracts (data not shown).

DISCUSSION

Elucidating the function of the PMP22 protein is important for understanding the mechanism by which both a duplication containing the PMP22 gene in humans and two point mutations in the PMP22 gene in mice may give rise to peripheral neuropathies. Toward this goal, we have begun to analyze PMP22 production in cultured Schwann cells in order to establish a suitable model system for studying the regulation and function of this protein. In this study, we have identified both similarities and differences between PMP22 expression in cultured Schwann cells, in intact peripheral nerve, and in purified myelin from young adult nerves.
Judged by SDS-PAGE, which is similar to that of PMP22 in f).

Peptide in amounts that are easily detected by metabolic to that in sciatic nerve and that they also produce PMP22 the N-linked carbohydrates. It is clear that PMP22 from

Cultured Schwann was preblocked with the respective peptide

Schwann cells exposed to antibodies to SlOO protein. To show the antibodies to peptide

The data show that cultured Schwann cells contain a 1.8-kilobase mRNA transcript coding for PMP22 identical in size to that in sciatic nerve and that they also produce PMP22 protein in amounts that are easily detected by metabolic labeling methods using radiolabeled methionine. Schwann cell-derived PMP22 has a molecular weight of 22,000, as judged by SDS-PAGE, which is similar to that of PMP22 in sciatic nerve segments labeled metabolically ex vivo and in isolated peripheral nerve myelin studied by Western blot analysis. In some studies, PMP22 migrated as a doublet (see Figs. 2, 4, 6, and 8) that appeared to arise from post-translational changes in glycosylation since in Figs. 6 and 8, the molecule migrated as an 18-kDa band following removal of the N-linked carbohydrates. It is clear that PMP22 from Schwann cells, intact sciatic nerve, and in purified myelin can be altered by agents that affect N-linked glycosylation. Metabolic labeling of Schwann cells and of sciatic nerve segments in medium containing tunicamycin yields an 18-kDa protein which is recognized by antibodies to PMP22 peptides. N-Glycanase treatment of PMP22 in purified sciatic nerve myelin produces the same 18-kDa protein. Pulse-chase studies carried out on Schwann cells suggest that the 18-kDa molecule is a short-lived precursor (half-life under 30 min) that is glycosylated to yield mature PMP22. This result is in full agreement with previous cDNA studies which predict that the core protein of PMP22 has a molecular weight of 18,000 (Welcher et al., 1991; Spreyer et al., 1991). In the absence of tunicamycin, the protein is synthesized in Schwann cells and in sciatic nerve segments with an apparent molecular weight of 22,000, the difference apparently being due to glycosylation. Taken together, these results support the notion that cultured Schwann cells produce a form of PMP22 similar to that in peripheral nerves.

Differences between PMP22 in intact nerve and in cultured Schwann cells were also detected, however. PMP22 expression is markedly lower in cultured Schwann cells than in intact nerves, even when production is stimulated by forskolin. Also, the turnover rate of PMP22 is rapid in cultured Schwann cells with a half-life of 30–60 min. Although the half-life of PMP22 in vivo has not been directly measured, it seems likely that its turnover rate in peripheral nerves is much slower as is the case for other protein constituents of compact myelin (Davison, 1961; Gould, 1977). Also, in peripheral nerves of aged animals, PMP22 mRNA levels are markedly diminished relative to levels of the protein, suggesting that once synthesized, PMP22 is not rapidly replaced (Snipes et al., 1992).

We also detected some antigenic differences between PMP22 synthesized by myelinating Schwann cells within explanted sciatic nerve segments and by passaged nonmyelinating Schwann cells in vitro. Antibody 1 raised against a synthetic peptide of PMP22, which contains a consensus site for N-linked glycosylation, failed to recognize PMP22 produced by cultured Schwann cells but did recognize the protein synthesized by Schwann cells in segments of sciatic nerve. This result suggests that subtle differences may exist in the nature of the carbohydrate moieties in PMP22 from the two sources. Antibody to peptide 1 recognized the 18-kDa unglycosylated form of the molecule synthesized by cultured cells and nerve segments in the presence of tunicamycin, suggesting that the antibody recognizes epitopes against which it was raised but not when the consensus site is glycosylated by cultured Schwann cells.

Antibody to peptide 1, but not peptide 2, specifically immunoprecipitated large amounts of a 48-kDa protein from...
the proteins resolved by 12% SDS-PAGE (20 pg/lane). Rat sciatic nerves by sucrose density gradient ultracentrifugation and samples stained with Coomassie Blue; myelin proteins, besides transfers stained with anti-PMP22 peptide 2 (1:2000) using the indirect peroxidase method developed using a chemiluminescence detection system.

PMP22 mRNA and protein are expressed in Schwann cell cultures at considerably lower levels than in Schwann cells in vivo in agreement with previous studies, indicating an important regulatory influence of the axon on the expression of a variety of Schwann cell genes. Rapid down-regulation of myelin-related genes in the absence of axons occurs in Schwann cells after nerve section in vivo or in Schwann cell cultures (Brockes et al., 1980; Mirsky et al., 1990; LeBlanc et al., 1987; Trapp et al., 1988; Toma et al., 1992; Lemke and Chao, 1988; Mitchell et al., 1990; Morrison et al., 1991). Conversely, axon-associated signals that promote protein synthesis in Schwann cells can partially and under specific circumstances be mimicked by agents that elevate intracellular cAMP levels (Baron-Van Evercooren et al., 1986; Sohue et al., 1986; Lemke and Chao, 1988; Shuman et al., 1988; Mirsky et al., 1990; Morgan et al., 1991). Forskolin and cAMP analogues, for instance, induce re-expression of Schwann cell-specific glycolipids, MBP, P₀ and $\text{P}_{190}k$ glycoprotein in cultured neonatal Schwann cells that fail to express these molecules once removed from axonal contact. Similarly, we report here that expression of PMP22 mRNA and protein is strongly up-regulated in cultured Schwann cells by the adenylate cyclase activator forskolin. Forskolin cannot, however, completely mimic the effect of axons on Schwann cells, since, as seen for other myelin proteins, levels of PMP22 mRNA and protein in forskolin-treated primary Schwann cells are significantly lower than in intact nerves.
PMP22 mRNA is chemically identical to growth arrest-specific-3 mRNA (gas3; Welcher et al., 1991), a transcript that has been associated with cellular growth arrest during the Go phase of the cell cycle in cultured fibroblasts (Schneider et al., 1988; Manfioletti et al., 1990). Given that PMP22 is up-regulated by forskolin in passaged Schwann cells in vitro, elevated cAMP levels may also up-regulate PMP22 expression in Schwann cell precursors in vivo, leading to cellular growth arrest and the differentiation of these cells into the myelinating Schwann cell phenotype. This regulatory mechanism might be reflected in the neurological mouse mutants $\text{T}r$ and $\text{T}r'$ which carry point mutations in the PMP22 gene (Suter et al., 1992a, 1992b). These defects in PMP22 are most likely responsible for the $\text{T}r$ and $\text{T}r'$ phenotype which is characterized by abnormal continuous Schwann cell proliferation throughout life and severe myelin deficiencies. It should be emphasized, however, that the available data are also consistent with the interpretation that PMP22 is not involved in Schwann cell growth regulation, but rather plays regulatory and functional roles which are related to the other protein components of compact peripheral nervous system myelin. Conceivably, PMP22 may serve two functions in Schwann cell development: as a regulator of Schwann cell division in the initial phase of peripheral nervous system development and as a structural component of myelin during maturation of the peripheral nervous system. Such alternating roles have also been suggested for other myelin proteins, based on embryonic expression patterns of DM-20 (Timsit et al., 1992; Ikenaka et al., 1992) and the effects of specific mutations in proteolipid protein (PLP; Schneider et al., 1992), the potential central nervous system counterpart of PMP22 (Snipes et al., 1992).

Previous studies have suggested that PMP22 is a myelin glycoprotein (Kitamura et al., 1981; Welcher et al., 1991), and in this study we confirm that PMP22 in myelin is N-glycosylated. Analyzed on SDS gels, PMP22 in purified myelin migrates as multiple bands, suggesting heterogeneous post-translational modifications. This finding is not unexpected for a myelin protein. For example, $\text{P}_0$ is glycosylated (Everly et al., 1973; Wood and Dawson, 1974; Smith and Sternberger, 1986).

![Fig. 9. Western blot analysis of PMP22 in purified myelin preparations. Myelin membranes were purified from young adult rat sciatic nerves by sucrose density gradient ultracentrifugation and the proteins resolved by 12% SDS-PAGE (20 pg/lane). A: lane 1, samples stained with Coomassie Blue; myelin proteins, besides PMP22, are identified by comparisons with published electrophoretic protein profiles of peripheral nerve myelin (Lees and Brostoff, 1984). Lane 2, an identical sample transferred to nitrocellulose and immunostained with antibodies to anti-PMP22 peptide 2 (1:2000 each) using the indirect peroxidase method developed using a chemiluminescence detection system. B, endoglycosidase treatment of the myelin membranes. The electrophoretic profiles of myelin membranes (20 pg of protein/lane) incubated overnight in the absence (B, lanes 1 and 3) or in the presence of N-glycanase (B, lanes 2 and 4) stained with Coomassie Blue (B, lanes 1 and 2) and parallel electrophoretic transfers stained with anti-PMP22 peptide 2 (1:2000) using the indirect peroxidase method developed using a chemiluminescence detection system (B, lanes 3 and 4). Results of these studies show that PMP22 in sciatic nerve, like in cultured Schwann cells, is a glycosylated protein that can be converted by deglycosylation into an 18-kDa core protein.](image-url)
1982), sulfated (Matthieu et al., 1975), phosphorylated (Singh and Spritz, 1976; Wiggins and Morell, 1980) and acylated (Agrawal et al., 1985). Several lines of evidence in this study (reviewed above) indicate that PMP22 in cultured Schwann cells, sciatic nerve, and purified myelin is glycosylated, but the identity of the side chains has not been clarified. Furthermore, the nature of the side chains may not be identical in PMP22 from the three sources since antibody to peptide 1, which is a segment of PMP22 containing a consensus sequence for N-linked glycosylation, recognized PMP22 in metabolically labeled segments of sciatic nerve but not in cultured Schwann cells. Identifying the side chains of PMP22 will be of considerable interest, since it seems likely, based on the localization of PMP22 in compact myelin and the nature and consequences of the PMP22 mutations in TR and TRd mice, that PMP22 plays some role in the compaction of the myelin sheath. N-Linked glycosylated groups on P0 have been implicated previously as a major determinant in the adhesive properties of this molecule (Filbin and Tennekoon, 1991; Yazaki et al., 1992).

In conclusion, results in this study show that PMP22 is expressed in expanded Schwann cell cultures, its production is regulated by forskolin, and the molecule is produced in a glycosylated form that appears to be generated from an 18-kDa precursor. Furthermore, a similar form of the protein is produced within sciatic nerve segments metabolically labeled ex vivo, and like other myelin proteins, PMP22 is a major glycoprotein constituent of isolated rat myelin. Our studies provide the basis for future in vitro experiments aimed at understanding the functional role of PMP22 in the myelination and growth regulation of normal and diseased peripheral nerves.

Acknowledgments—We thank Cheryl Richards and Julie Haskins for excellent technical assistance and Drs. Vera Chiumecky, Manijeh Pasdar, and Gordon Sauvé for frequent discussions and advice. We also thank Jaclyn Peebles and Greg Morrison for help in preparing the figures.

REFERENCES

Agrawal, H. C., Schmidt, R. E., and Agrawal, D. (1983) J. Biol. Chem. 258, 6556-6560.
Barker, P. A., Miller, F. D., Large, T. H., and Murphy, R. A. (1991) J. Biol. Chem. 266, 19113-19119.
Baron-Van Evercooren, A., Gansmuller, A., Gumpel, M., and Quarles, R. H. (1991) J. Neuropath. Exp. Neurol. 50, 293-302.
Brockes, J. P., and Vogelstein, B. (1987) Mol. Brain Res. 2, 182-196.
De Leval, M., Welcher, A. A., Suter, U., and Shooter, E. M. (1987) J. Neurosci. 7, 437-448.
Everly, J. L., Brady, R. O., and Quarles, R. H. (1973) J. Neurochem. 21, 329-334.
Feinberg, A. P., and Vogelstein, B. (1984) Annu. Rev. Biochem. 53, 217-267.
Filbin, M. T., and Tennekoon, G. I. (1989) J. Biol. Chem. 264, 85-85.
Agrawal, H. C., Schmidt, R. E., and Agrawal, D. (1983) J. Biol. Chem. 258, 6556-6560.
Barker, P. A., Miller, F. D., Large, T. H., and Murphy, R. A. (1991) J. Biol. Chem. 266, 19113-19119.
Baron-Van Evercooren, A., Gansmuller, A., Gumpel, M., and Quarles, R. H. (1991) J. Neuropath. Exp. Neurol. 50, 293-302.
Brockes, J. P., and Vogelstein, B. (1987) Mol. Brain Res. 2, 182-196.
De Leval, M., Welcher, A. A., Suter, U., and Shooter, E. M. (1987) J. Neurosci. 7, 437-448.
Everly, J. L., Brady, R. O., and Quarles, R. H. (1973) J. Neurochem. 21, 329-334.
Feinberg, A. P., and Vogelstein, B. (1984) Annu. Rev. Biochem. 53, 217-267.
Filbin, M. T., and Tennekoon, G. I. (1989) J. Biol. Chem. 264, 85-85.
Agrawal, H. C., Schmidt, R. E., and Agrawal, D. (1983) J. Biol. Chem. 258, 6556-6560.
Barker, P. A., Miller, F. D., Large, T. H., and Murphy, R. A. (1991) J. Biol. Chem. 266, 19113-19119.
Baron-Van Evercooren, A., Gansmuller, A., Gumpel, M., and Quarles, R. H. (1991) J. Neuropath. Exp. Neurol. 50, 293-302.
Brockes, J. P., and Vogelstein, B. (1987) Mol. Brain Res. 2, 182-196.
De Leval, M., Welcher, A. A., Suter, U., and Shooter, E. M. (1987) J. Neurosci. 7, 437-448.
Everly, J. L., Brady, R. O., and Quarles, R. H. (1973) J. Neurochem. 21, 329-334.
Feinberg, A. P., and Vogelstein, B. (1984) Annu. Rev. Biochem. 53, 217-267.
Filbin, M. T., and Tennekoon, G. I. (1989) J. Biol. Chem. 264, 85-85.
Agrawal, H. C., Schmidt, R. E., and Agrawal, D. (1983) J. Biol. Chem. 258, 6556-6560.
Barker, P. A., Miller, F. D., Large, T. H., and Murphy, R. A. (1991) J. Biol. Chem. 266, 19113-19119.
Baron-Van Evercooren, A., Gansmuller, A., Gumpel, M., and Quarles, R. H. (1991) J. Neuropath. Exp. Neurol. 50, 293-302.
Brockes, J. P., and Vogelstein, B. (1987) Mol. Brain Res. 2, 182-196.
De Leval, M., Welcher, A. A., Suter, U., and Shooter, E. M. (1987) J. Neurosci. 7, 437-448.
Everly, J. L., Brady, R. O., and Quarles, R. H. (1973) J. Neurochem. 21, 329-334.
Feinberg, A. P., and Vogelstein, B. (1984) Annu. Rev. Biochem. 53, 217-267.
Filbin, M. T., and Tennekoon, G. I. (1989) J. Biol. Chem. 264, 85-85.
Agrawal, H. C., Schmidt, R. E., and Agrawal, D. (1983) J. Biol. Chem. 258, 6556-6560.