Lysosomal Delivery of the Major Myelin Glycoprotein in the Absence of Myelin Assembly: Posttranslational Regulation of the Level of Expression by Schwann Cells

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Abstract. The major myelin protein, P0, has been shown to have decreased levels of expression and altered oligosaccharide processing after the disruption of Schwann cell-axon interaction. We show here that lysosomal degradation of the glycoprotein shortly after its synthesis accounts for much of its decreased expression in the permanently transected adult rat sciatic nerve, a denervated preparation where there is no axonal regeneration or myelin assembly. If [3H]mannose incorporation into sciatic nerve endoneurial slices is examined in the presence of the lysosomotropic agent, NH4Cl, a marked increase in the level of newly synthesized P0 is seen. Pulse-chase analysis of [3H]mannose-labeled P0 in the presence of NH4Cl indicates that this increase is a consequence of inhibition of P0 degradation that normally occurs 1–2 h after biosynthesis in the transected nerve. P0 degradation can also be inhibited if lysosomal function is disturbed by dilution of secondary lysosomes with L-methionine methyl ester. The addition of deoxymannonojirimycin or swainsonine (SW), inhibitors of oligosaccharide-processing mannosidases I and II, respectively, also results in a decrease in P0 degradation. This inhibition is presumably caused by a blockage of transport to the lysosomes due to altered processing of the glycoprotein, although the direct inhibition of lysosomal mannosidases cannot be excluded. In contrast to the transected nerve, addition of NH4Cl or SW has no effect on P0 levels in the crushed nerve, where myelin assembly occurs. The delivery of P0 to the lysosomes of the transected nerve Schwann cells does not appear to be triggered by the mannose-6-phosphate transport system involved in acid hydrolase routing. The accumulation of a fucosylated species of P0 in the presence of SW indicates that the glycoprotein has progressed at least as far as the site of GlcNAc transferase I without lysosomal delivery, and thus differs from the typical mannose-6-phosphate-containing glycoproteins. Furthermore, there is no evidence of P0 phosphorylation either in the absence or presence of NH4Cl or SW. These findings indicate that the amount of P0 in the transected nerve is regulated at the posttranslational level as well as at the level of transcription.

The intracellular sorting of proteins from their site of biosynthesis to their final subcellular location is a topic that has received considerable attention. Current evidence points to two general types of protein movement: that involving specific recognition of newly synthesized proteins, presumably via interaction with receptor-like molecules (regulated transport), and that involving passive, bulk flow that does not rely on the recognition of the transported protein (constitutive transport) (15, 21, 44). Receptor-mediated intracellular targeting has been directly demonstrated in the delivery of hydrolases to the lysosomes (5, 16, 43). Poruchynsky et al. (33) have recently shown that the deletion of an NH2-terminal hydrophobic region of a rotavirus glycoprotein normally confined to the ER results in the constitutive secretion of the protein. This suggested to the authors that the missing sequence might have served as a signal specifying ER localization. Another example of a signal that specifies an ER destination can be seen with the NH2-
terminal signal sequences found on integral membrane and secretory proteins (42). These sequences have been shown to interact with a recognition particle that aids in the binding of the ribosome–protein complex to the ER membrane (51). Cleavable peptide sequences also exist on mitochondrial proteins that seem to play a role in targeting the molecules to the respiratory organelle (42). Obviously, the identification of more recognition systems involved in intracellular transport will greatly aid in the understanding of how proteins reach their final destinations.

Our laboratory has become interested in the mechanisms of control of intracellular transport since we discovered that rat peripheral nerve Schwann cells respond to injury by altering the levels and posttranslational processing of the major myelin protein, P₀ (18, 26, 27, 31). In normal adult rat sciatic nerve, the P₀ molecule is an integral myelin membrane glycoprotein that contains a single complex type asparagine-linked oligosaccharide chain that is fucosylated and sulfated (20, 52). If the adult rat sciatic nerve is crush-injured, axonal degeneration and demyelination occur, ultimately resulting in Schwann cell proliferation, nerve fiber regeneration, and remyelination (26). This remyelination process is accompanied by increased production of P₀ having the mature, complex-type oligosaccharide chain (27). The level of expression and the extent of oligosaccharide processing of P₀ have been shown to be drastically changed in nerve injury models where there is no axonal contact and subsequently no myelin assembly, such as the permanently transected nerve and cultured neonatal Schwann cells (18, 27, 29, 31, 32). After permanent nerve transection, the majority of the P₀ molecules are found to contain the immature Man₉GlcNAc₂ oligosaccharide chain, although a small amount of this intermediate species matures to the complex type oligosaccharide chain (27). The accumulation of the high-mannose form of the glycoprotein suggests that this species of the molecule is not exposed to the mannosidases involved in further processing, with slow transport of the molecule from the ER to the Golgi being a possible cause of this inaccessibility to the trimming glycosidases. If a pulse–chase analysis of P₀ is performed with transected nerve, it can be shown that complex type chain is formed at a slow rate (>1 h) but that the total level of P₀ observed after 1–2 h of chase is less than the original amount of high-mannose P₀ seen during the pulse (27). This suggests that much of the newly synthesized P₀ is being retargeted to a site of intracellular degradation. That the transected nerve Schwann cell would want to rid itself of P₀ is not necessarily surprising since the glycoprotein cannot be assembled into myelin in the absence of axonal contact (32).

Two general methods exist for the degradation of endogenous cellular proteins: (a) many short-lived proteins or proteins with structural abnormalities are degraded via the ATP-dependent ubiquitin pathway (3) while (b) longer-lived proteins seem to be degraded by lysosomal hydrolyses (13). Since P₀ is in a membrane environment after its synthesis on ER-bound polysomes, accessibility to the former degradative system seems unlikely. We have thus decided to examine whether P₀ is being retargeted to lysosomes and subsequently degraded in the transected nerve. The results of this investigation, which follow, indicate that the level of P₀ in the transected nerve is indeed regulated posttranslationally by lysosomal catabolism. Furthermore, this delivery to the lysosomes appears to occur by a method other than the typical mannose-6-phosphate recognition system, as high-mannose forms of the glycoprotein do not exit to the lysosomes. The lack of observable P₀ phosphorylation in the transected nerve further implies that this modification does not play a role in the targeting of the glycoprotein.

Materials and Methods

Preparation of Permanently Transected and Crushed Adult Rat Sciatic Nerve

Sciatic nerves from male adult Sprague–Dawley rats (~200 g) were permanently transected or crushed as described previously (26). Briefly, rats were anesthetized with sodium pentobarbital, and their sciatic nerves were exposed below the sciatic notch and ligated by tying two sutures around the nerve trunk. The nerve was cut between the sutures and each end was repositioned by 180° and tied to adjacent muscle. This permanent nerve transection prevents the reentry of axons from the proximal nerve segment into the distal segment and hence subsequent remyelination. Crushed injured nerves were prepared by crushing sciatic nerves below the sciatic notch with a pair of fine forceps. The crushed nerves exhibit axonal regeneration and remyelination of the site distal to injury, after an initial degeneration stage. After closure of the wounds, the animals were maintained for 35–37 d. The nerves were removed for incorporation studies by anesthetizing the animals with sodium pentobarbital and excising the distal segment of the sciatic nerve, including the tibial, peroneal, and sural branches. Appropriate precautions were taken for the proper care of the animals according to the standards established by the Animal Welfare Acts and the "NIH Guide for the Care and Use of Laboratory Animals" (No. 85–23 Revised 1985). Laboratory animal use was approved by the Mayo IACUC.

Precursor Incorporation into Sciatic Nerve Endoneurial Slices

The microdissection technique of Dyck et al. (9) was used to remove the endoneurium from the perineurium and epineurium of sciatic nerves 35–37 d post-injury. The endoneurium from two or more nerves was sliced into 2-mm pieces and pooled together in modified Krebs-mammalian Ringer's solution unless otherwise noted, with subsequent splitting of the pooled slices into equal-sized portions for incubation with precursors. For incorporation of D-[2,6-3H]mannose (54 Ci/mmol; Amersham Corp., Arlington Heights, IL), endoneurial slices were added to 100–250 μCi of the isotope in 0.5 ml of Krebs-Ringer's solution, pH 7.4, and incubated at 37°C in an atmosphere of 95% O₂–5% CO₂ for the times indicated in the figure legends. All comparative studies were incubated with equal amounts of isotope. 10 mM pyruvate was used as an energy source instead of glucose, since the latter binds to mannose receptors (27). In the cases where the endoneurial slices were chased after precursor incorporation, the pulse solutions were replaced with Krebs-Ringer's solution containing 10 mM glucose or 10 mM mannose and incubated for the times indicated in the figure legends. Incorporations carried out in the presence of inhibitors were as above, except that 10 mM NH₄Cl, 10 mM L-isoleucine methyl ester, 4 mM deoxy-mannojirimycin (dMM), or 10 or 100 μM SW were added as indicated. All chases and washes of samples that were incorporated with inhibitors contained an equal concentration of inhibitor. For the incorporation of [1,5,6-3H]fucose (45 Ci/mmol, Amersham Corp.), H-amino acid mixture (1 mCi/ml, Amersham Corp.), and [32P]orthophosphate (carrier free, 285 Ci/mg; ICN Biochemicals, Inc., Irvine, CA), endoneurial slices were placed in 0.5 ml of Krebs-Ringer's solution containing 11.5 mM glucose and 200–250 μCi of the isotopes. Comparative studies were incubated with equal amounts of isotope. Incubations were carried out as above for the times indicated in the figure legends either in the absence or in the presence of 10 mM NH₄Cl, 10 mM L-isoleucine methyl ester, or 10 or 100 μM SW. Pulse solutions were replaced before chase with Krebs-Ringer's solution containing 10 mM fucose or amino acid supplement (KC Biological Inc., Lenexa, KS) in place of [3H]fucose and H-amino acid mixture, respectively.

Endoneurial Fractionation and Solubilization

The endoneurial slices were fractionated according to the procedure of Poduslo (26). Briefly, the slices were homogenized in ice-cold distilled wa-
obtained, which was then centrifuged in an airfuge 30 ° rotor at 197,000 g at 4°C (model A-100; Beckman Instruments, Fullerton, CA). The supernatant obtained from this centrifugation was designated as the S-I fraction. The resulting pellet was reconstituted in water, and SDS was added to a final concentration of 1.25%. This mixture was sonicated for 1 h in an ultrasonic cleaner (model B-220; Branson Sonic Power, Inc., Danbury, CT), and then centrifuged as above. The supernatant from this step was the S-II fraction. In some cases, the endoneurial homogenates had SDS added before a first centrifugation. These samples were sonicated and centrifuged as above, with the resulting supernatant serving as a combined S-I and S-II fraction. Aliquots were taken from the fractions and assayed for protein according to the procedure of Lowry et al. (19) as modified by Hess and Lewin (14), using monomer BSA (Miles Laboratories, Inc., Elkhart, IN) as a standard.

**SDS-Pore Gradient Electrophoresis (SDS-PGE)**

Endoneurial proteins were separated by SDS-PGE on a linear gradient with a gel concentration of 10–20% T and 1% C, using a programmable ultragradient maker (LKB Instruments, Inc., Gaithersburg, MD) according to a previously described procedure (25). A Tris-glycine buffer system was used. Proteins were solubilized in a solution containing 1% SDS (wt/vol); 0.0625 M Tris, pH 6.8; 5% 2-mercaptoethanol (vol/vol); 0.002% bromophenyl blue (wt/vol); and 10% glycerol (vol/vol). 2-Mercaptoethanol was omitted from solubilizing solutions where noted in the figure legend. Protein samples of 2.5–15 μg were subjected to electrophoresis for 2.5–3 h at a constant power of 20 W (model 3-1500 power supply; Buchler Instruments, Fort Lee, NJ). The gels and surrounding buffer were cooled to 0.5°C with a refrigerated circulating bath (model RTE 8; NESLAB Instruments, Inc., Portsmouth, NH). After electrophoresis, the gels were fixed in methanol/water/acetic acid (45:45:10). Gels containing 1% SDS samples were treated with 20% 2,5-diphenyloxazole (PPO) (wt/vol) in glacial acetic acid (34), followed by removal of the PPO solution and precipitation of the gel-embedded fluor with 5% (vol/vol) glycerol. The gels containing 3H samples were treated with the glycerol solution immediately after fixation. Gels were dried on a gel drier (model SE-1150; Hoefer Scientific Instruments, San Francisco, CA) before exposure to Kodak X-Omat AR or S film. Molecular weight estimates were determined by evaluating the relationship log (M) versus log (R) as described previously (28), except that the estimates were made from fluorograms using 14C-methylated protein standards (Amerham Corp.).

**Immunoprecipitation of P0**

Immunoprecipitation of P0 was done as previously described (27). Briefly, aliquots from the S-I fraction of normal, crushed, and transected nerves (15-μg samples) that had been incorporated with [3H]orthophosphate were incubated with 7.5 μl of rabbit anti-chick P0 gamma-globulin (kindly provided by Dr. Catherine Mezei, Dalhousie University, Nova Scotia, Canada) (23) in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, for 20 h at 4°C. The immunoprecipitate was obtained by centrifugation at 197,000 g at 4°C in an airfuge 30 ° rotor (model A-100; Beckman Instruments, Inc.). Precipitates were washed twice with H2O followed by centrifugation, and the proteins of the immunoprecipitate were solubilized in solubilizing solution and analyzed by SDS-PGE.

**Results**

**Effects of Lysosomal Inhibitors on P0 Levels**

To investigate whether P0 is routed to the lysosomes in the transected nerve Schwann cell, the effect of the lysosomotropic agent NH4Cl was examined. Weak bases such as NH4Cl inhibit hydrolyses by elevating intralysosomal pH to a level that is outside the range of enzyme activity (41). In addition to direct inhibition of lysosomal hydrolase, NH4Cl can also indirectly block catabolism by impairing receptor-mediated shuttling of ligands to the lysosomes by inhibiting receptor-ligand uncoupling (1). As seen in Fig. 1, addition of NH4Cl to transected nerve preparations results in a dramatic increase in the levels of [3H]mannose incorporated into newly synthesized P0 in the S-I and S-II fractions. Poduslo and Yao (30) have shown that the S-I fraction contains P0 in a lipid environment, presumably in micelles or membrane vesicles formed from cellular membranes during the hypotonic homogenization of the nerve endoneurium. The S-II fraction contains the remainder of the membrane inserted P0 not extracted into the S-I fraction.

In addition to the high-mannose type species normally seen in the transected nerve, a new, faster migrating species of P0 with an M, of 27,100 is formed after the addition of NH4Cl. This species shows a shift in M, of ~4,000 when analyzed by SDS-PGE in the absence of reducing agent (Fig. 2), a property previously shown to be characteristic of P0 (26, 31). The effect of NH4Cl on the levels of P0 is specific for the transected nerve, as no increase in the levels of the glycoprotein are seen in crushed nerve preparations treated with the lysosomotropic agent (Fig. 1).

The increase in the levels of P0 observed after the addition of NH4Cl can be shown to be due to an inhibition of P0 degradation. As seen in Fig. 3, pulse–chase analysis of [3H]mannose-labeled P0 reveals that the glycoprotein is degraded 1–2 h after synthesis in the transected nerve. This degradation is prevented when NH4Cl is present in the pulse and chase media (Fig. 3). Although the effect of NH4Cl is presumably due to inhibition of lysosomal degradation (41), it has been shown that weak amines can also alter terminal

**Figure 1.** [3H]Mannose incorporation into permanently transected and crushed adult rat sciatic nerve endoneurial slices in the absence and presence of NH4Cl. Permanently transected and crushed endoneurial slices were incubated with [3H]mannose for 3 h in the absence and presence of NH4Cl, with subsequent sample preparation to obtain S-I and S-II fractions. Each incubation was performed with endoneurial slices from a single nerve. 2.5 μg of total protein from each sample fraction was analyzed by SDS-PGE, followed by fluorography. A 4C-protein standard mixture was also included on the gel. The gel was exposed to Kodak X-Omat S film at -70°C for 14 d.

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known at this time. As with the 27.1-kD species of P0 generated after NH4Cl treatment, the lower M, species formed in chase with L-methionine methyl ester shows a shift of 4 kD on SDS-PGE in the absence of reducing agent and can be immunoprecipitated with anti-P0 antibody (data not shown). Pulse–chase analysis of [3H]-amino acid-labeled P0 in the absence of the lysosomal inhibitor reveals substantial P0 degradation within 2 h after synthesis (Fig. 4), coinciding with the results seen when [3H]mannose is used as precursor. This indicates that the polypeptide chain and oligosaccharide moiety of P0 are processed in the lysosomes within a similar time span.

**Effects of Processing Inhibitors on P0 Degradation**

Addition of SW, a Golgi mannosidase II and lysosomal α-mannosidase inhibitor (40, 46), also results in an increased level of [3H]mannose-labeled P0 in transected nerve preparations (Fig. 5). The effect of SW on the intracellular level of P0 can also be demonstrated by using a 3H-amino acid mixture to visualize de novo synthesis of the glycoprotein (Fig. 5). A dramatic increase in the amount of the glycoprotein poly-

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**Figure 2.** The electrophoretic mobility of P0 labeled with [3H]mannose in the presence and absence of 2-mercaptoethanol (2-ME). 2.5 µg of total protein from S-I fractions of transected nerve endoneurial slices labeled for 3 h with [3H]mannose in the absence and presence of NH4Cl were analyzed by SDS-PGE, followed by fluorography. The samples were solubilized before electrophoresis in solubilizing buffer with or without 5% 2-mercaptoethanol. A ¹⁴C-protein standard mixture was also included on the gel, which was exposed to Kodak X-Omat S film at -70°C for 14 d. Intervening lanes were removed before photography.

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**Figure 3.** Pulse–chase analysis of the incorporation of [3H]mannose into permanently transected endoneurial slices in the absence and presence of NH4Cl. 15-µg samples of total protein from combined S-I and S-II fractions were analyzed by SDS-PGE, followed by fluorography. Incubations of endoneurial slices with [3H]mannose were allowed to proceed for 45 min in the absence and presence of NH4Cl, followed by chases of 60 and 120 min in the absence and presence of the inhibitor. The migrations of ¹⁴C-protein standards are indicated. The gel was exposed to Kodak X-Omat S film for 14 d at -70°C. Intervening lanes were removed before photography.
Figure 4. Pulse-chase analysis of the incorporation of 'H-amino acid mixture into transected endoneurial slices in the absence and presence of L-methionine methyl ester (L-Met-OMe). 15-μg samples of total protein from combined S-I and S-II fractions were analyzed by SDS-PGE, followed by fluorography. Incubations of endoneurial slices were allowed to proceed for 1 h in the absence and presence of L-Met-OMe, followed by a 2-h chase in the absence and presence of inhibitor. A 14C-protein standard mixture was included on the gel, which was exposed to Kodak X-Omat AR film for 3 d at -70°C.

Figure 5. [3H]Mannose and 'H-amino acid mixture incorporation into permanently transected endoneurial slices in the absence and presence of SW. A montage of two SDS-PGE gels is shown. 15-μg samples of total protein from combined S-I and S-II fractions of a 6-h incorporation of [3H]mannose (with or without SW), and 10-μg samples of total protein from S-I and S-II fractions of a 3-h incorporation of 'H-amino acid mixture (with or without SW) were analyzed. Each [3H]mannose incorporation was done with endoneurial slices from a single nerve. A 14C-protein standard mixture was included on the gels, which were exposed to Kodak X-Omat AR film at -70°C for 3 d.

Figure 6. Pulse-chase analysis of the incorporation of [3H]mannose into permanently transected endoneurial slices in the absence and presence of SW. 15-μg samples of total protein from combined S-I and S-II fractions were analyzed by SDS-PGE, followed by fluorography. Incubations of endoneurial slices were allowed to proceed for 1 h in the absence and presence of SW, followed by a 2-h chase in the absence and presence of inhibitor. A 14C-protein standard mixture was included on the gel, which was exposed to Kodak X-Omat AR film for 5 d at -70°C. Intervening lanes were removed before photography.

peptide chain can be seen in both the S-I and S-II fractions after incorporation in the presence of this inhibitor. The identity of the 'H-amino acid-labeled protein seen after SW treatment has been verified as being P0 through immunoprecipitation with anti-P0 antibody (data not shown). As is the case with NI-14C1, addition of SW does not cause a significant increase in the level of P0 in the crushed nerve (data not shown).

The effect of SW on the amount of P0, like that of the lysosomal inhibitors, is a consequence of its prevention of P0 degradation. Fig. 6 demonstrates that SW blocks [3H]mannose-labeled P0 catabolism when it is included in pulse-chase media, whereas a similar treatment in the absence of SW results in P0 degradation after 2 h of chase. SW may be inhibiting P0 degradation by blocking the action of the lysosomal α-mannosidas, as it has been demonstrated that removal of the oligosaccharide moiety from glycoproteins is often a prerequisite to further protein catabolism (40). Alternatively, SW may inhibit the formation of a species of P0 that is destined for degradation after it is formed subsequent to the action of Golgi GlcNAc transferase I. This would result in a buildup of P0 in the cell that is not recognized as being targeted to the lysosomes, with this P0 presumably containing a hybrid-type oligosaccharide chain (47). Although the possibility that SW exerts its effect on the lysosomes directly cannot be eliminated, it can be shown that the P0 generated in the presence of this inhibitor does progress to (and possibly beyond) the site of Golgi GlcNAc transferase I. As shown in Fig. 7, SW treatment results in the formation of a species of P0 that is fucosylated. This is in sharp contrast to the situation observed in untreated transected nerve, where only
Figure 7. [3H]Fucose incorporation into permanently transected endoneurial slices in the presence and absence of SW. 15-μg samples of total protein from combined S-I and S-II fractions were analyzed by SDS-PGE, followed by fluorography. Incubations of endoneurial slices were allowed to proceed for 3 h in the presence and absence of SW, followed by a 1-h chase in the presence and absence of inhibitor. A [3C]-protein standard mixture was included on the gel, which was exposed to Kodak X-Omat S film for 7 d at -70°C. Intervening lanes were removed before photography.

The previous section revealed that P0 that accumulates after SW treatment is fucosylated, indicating that the glycoprotein has progressed to the medial-Golgi and has been modified by Golgi mannosidase II in oligosaccharide processing (17). If P0 degradation is occurring after the action of Golgi mannosidase II, one would expect that any agent that blocks an oligosaccharide processing reaction before these enzymes would have an inhibitory effect on P0 catabolism. This is indeed the case, as addition of the Golgi mannosidase I inhibitor dMM (11) prevents P0 degradation. Fig. 8 documents that [3H]mannose-labeled P0 formed during a 1-h pulse remains after 2 h of chase in the presence of dMM. As shown previously and again in Fig. 8, [3H]mannose-labeled P0 is degraded after 2 h of chase in the absence of inhibitor.

Analysis of Mannose-6-Phosphate Involvement in P0 Lysosomal Delivery

The experiments with the lysosomal and processing inhibitors clearly demonstrate that P0 is normally routed to the lysosomes for degradation in the transected nerve. A mechanism whereby a cell directs an integral membrane (myelin) protein to a new cellular location (i.e., lysosomes) shortly after its synthesis is intriguing. It is known that lysosomal hydrolases are targeted to their acidic organelles by the use of a unique receptor–ligand system. The lysosomal enzymes are modified with phosphate groups on mannose residues of their oligosaccharide chains (16), allowing them to be recognized in the Golgi by a receptor for mannose-6-phosphate (5, 43).

The previous section revealed that P0 that accumulates after SW treatment is fucosylated, indicating that the glycoprotein has progressed to the medial-Golgi and has been modified by GlcNAc transferase I (38, 39). This finding strongly suggests that P0 is not transported by the usual mannose-6-phosphate system used in delivery of acid hydrolases. These enzymes are modified while in the high-mannose form (4, 22, 48, 49), before the action of GlcNAc transferase I. Although it has been reported that a fraction of phosphorylated hydrolases can progress to the site of GlcNAc transferase I, this class of molecule does not bind efficiently to the mannose-6-phosphate receptor (49). The possibility remains that P0 is transported by another ligand–receptor system that also relies on a phosphorylation event. To investigate this possibility, transected nerves were incubated in the presence of [32P]orthophosphate, and the labeling of P0 was examined. Phosphate labeling was also performed in the presence of NH4Cl and SW to ensure that the glycoprotein would accumulate. As seen in Fig. 9, labeling of P0 cannot be demonstrated in any of the transected nerve incubations. This lack of phosphate incorporation is in sharp contrast to the situation observed in normal and crush-injured nerves, where P0 is phosphorylated by a protein kinase after assembly into the myelin (2). As seen in Fig. 9, immunoprecipitation of S-I fractions from nerve slices incubated with [32P]orthophosphate results in the identification of phosphate-labeled P0 in the normal and crushed nerves, but not in the transected nerve. The identity of the 90-kD species that...
copolymers with $P_0$ in the normal and crushed nerves is unknown. That phosphorylation of $P_0$ cannot be demonstrated in transected nerves even when $P_0$ accumulates due to the presence of NH$_4$Cl and SW suggests that $P_0$ is targeted to the site of degradation by a mechanism that does not involve the use of phosphate groups.

**Discussion**

The mechanisms whereby newly synthesized proteins are targeted to their final intracellular destinations have not been fully elucidated to date. Although a few examples of targeting signals exist, such as the mannose-6-phosphate groups of lysosomal hydrolases (5, 16, 43), the majority of cellular proteins reach their proper destinations by unknown pathways and recognition systems. Earlier hypotheses that postulated that the oligosaccharide moieties of glycoproteins encoded routing signals have been proven to be invalid in many cases (42) since blocking glycosylation with tunicamycin, an inhibitor of dolichol-linked oligosaccharide synthesis, did not prevent proper targeting. The roles of protein sequence and structure in cellular targeting have also been addressed. Recent studies that have used molecular biology techniques to produce altered primary structures in membrane proteins have demonstrated that hydrophobic regions of proteins are necessary for proper membrane insertion (6, 37). Likewise, the formation of hybrid proteins that contain a secretory protein linked to a portion of an integral membrane protein indicates that hydrophobic sequences can anchor proteins that are normally soluble (7, 36, 55). It is not clear from these studies whether these anchoring portions of the proteins function in targeting, or instead function solely on the basis of their hydrophobic interaction with lipid bilayers. Further demonstration of targeting or retargeting events within cells would expand the understanding of the processes involved in determining the destination of cellular components.

The permanently transected rat sciatic nerve appears to be a model system that allows the investigation of retargeting mechanisms. The Schwann cells of this injury model show dramatic cellular alterations after injury, presumably as a consequence of inhibition of axonal contact. Recent studies indicate an approximately threefold decrease in the mRNA levels of the major myelin glycoprotein, $P_0$, after transection (18). Earlier studies demonstrated a change in oligosaccharide processing of $P_0$ after transection (27), with the cells showing an accumulation of an intermediate high-mannose species of the glycoprotein. This buildup of high-mannose type $P_0$ indicates that the rate of normal oligosaccharide processing is greatly reduced, presumably due to a decreased rate of transit from the ER to the Golgi. Alternatively, the altered processing may be the result of decreased kinetics of a Golgi mannosidase (27). The studies presented here reveal that the majority of the $P_0$ in the transected nerve is routed to the lysosomes after biosynthesis, apparently as a means of posttranslational regulation of the level of this myelin component in the absence of myelin assembly.

If transected nerve endoneurial slices are incubated for 3 h with [H$^3$]mannose in the presence of the lysosomotropic agent, NH$_4$Cl, one sees a substantial increase in the level of newly synthesized $P_0$. In addition to the typical high-mannose form of the glycoprotein usually seen in the transected nerve, a new species of glycoprotein with an $M_r$ of 27.1 kD is also formed. This species shows a shift in $M_r$ of 4 kD when analyzed by SDS-PGE in the absence of reducing agent, a property shown to be characteristic of $P_0$ (26, 31). Pulse-chase analysis reveals that the increase in $P_0$ levels in the presence of NH$_4$Cl is a consequence of inhibition of degradation of the glycoprotein that occurs 1–2 h after its synthesis. When degradation is blocked by the addition of NH$_4$Cl, both the high-mannose species and the 27.1-kD species appear after 1–2 h of chase. The latter species of $P_0$ may be the form targeted for the lysosomes or it may be a slightly degraded form of the glycoprotein that results from incomplete lysosomal inhibition by NH$_4$Cl. Definitive statements about this 27.1-kD species of $P_0$ await further characterization of the molecule.

Since weak amines such as NH$_4$Cl elevate the pH of acidic organelles, other aspects of cellular function may be altered by their presence. It has been shown that trans-Golgi processing (45) and receptor-mediated endocytosis (53) are disrupted by the addition of amines. To ensure that the effect of NH$_4$Cl was solely due to its inhibition of lysosomal function, an independent method of lysosomal disruption was evaluated. Amino acid methyl esters such as t-methionine methyl ester have been demonstrated to cause selective dilution of secondary lysosomes with no aberration of ER of Golgi structure (35). In addition, these agents alter lysosomal function through osmotic effects, with no appreciable change in lysosomal pH (8, 35). Thus, t-methionine methyl ester can be expected to inhibit lysosomal function without causing the pH-mediated side effects of the weak amines. As was the case with NH$_4$Cl, treatment of transected nerve
with L-methionine methyl ester results in the inhibition of P0 degradation. This confirms the role of lysosomes in P0 degradation within the transected nerve Schwann cells.

The means by which P0 is shunted to the lysosomes in transected nerve is unknown, but a potential mechanism for P0 delivery would be one that employed the mannose-6-phosphate system used in the transport of acid hydrolases to lysosomes (5, 10, 12, 16, 43). In this system, the acid hydrolases are phosphorylated on one or two of their α1,2-linked mannose residues (4, 16, 22, 48, 49), presumably before the action of Golgi mannosidase I (4). These modified glycoproteins are then bound by a receptor that is specific for the mannose-6-phosphate groups and routed to the lysosomes (5, 43). The role of the mannose-6-phosphate system in P0 targeting was investigated by using oligosaccharide processing inhibitors to obtain information concerning the oligosaccharide structure of the P0 species transported to the lysosomes. Addition of the Golgi mannosidase I inhibitor dMM (II) resulted in an inhibition of P0 lysosomal degradation. This implies that P0 is routed to the degradative organelles at a stage in oligosaccharide processing after the action of Golgi mannosidase I. This conclusion can be affirmed by examination of the effects of the Golgi mannosidase II and lysosomal mannosidase inhibitor, SW, on P0 catabolism. Addition of SW to transected nerve results in the inhibition of P0 degradation and the accumulation of a P0 species that is fucosylated. The inhibition of P0 degradation by SW could occur in two different ways. The most obvious mechanism of inhibition is one that results from the oligosaccharide chain of P0 not being recognized as targeted for the lysosomes because of inhibition of manniosidase II oligosaccharide processing. An alternative scheme relies on SW inhibition of lysosomal mannosidases that are involved in the catabolism of P0. This would result in a lysosomal buildup of the fucosylated glycoprotein that has been acted upon by GlcNAc transferase I and Golgi fucosyltransferase (38, 39). This latter type of inhibition would only result in a cessation of P0 degradation if oligosaccharide catabolism was a prerequisite to polypeptide degradation (40).

Regardless of which of these mechanisms is causing the inhibition of P0 catabolism by SW, the data reveal that the glycoprotein that is destined for the lysosomes is formed sometime after the action of Golgi GlcNAc transferase I. This implies that P0 has not been acted upon by the mannose-6-phosphate delivery system, as the enzymes involved in the targeting of acid hydrolases would have modified and transported the high-mannose oligosaccharides that are formed during dMM treatment and that are temporarily present during SW treatment.

Further evidence to support the contention that P0 is delivered to lysosomes without using the mannose-6-phosphate signal is that P0 does not appear to be phosphorylated in the transected nerve. This observation is made when the incorporation of [32P]orthophosphate is examined either in the absence or presence of NH4Cl and SW. This is in contrast to the situation observed in normal and crushed nerve, where P0 phosphorylation is readily visualized after the glycoprotein is assembled into myelin. Although the method of P0 delivery to the lysosomes is not fully elucidated at this time, it is clear that a mechanism unlike that used by acid hydrolases is functioning. It is known that other transport methods exist, since I-cell disease hydrolases can reach the lysosomes in the absence of a functioning mannose-6-phosphate system (24, 50).

In summary, it appears that the lack of axonal contact resulting from permanent nerve transection causes Schwann cells to drastically alter their cellular processing of the major myelin glycoprotein, P0. In addition to changes in transcription (18) and posttranslational processing (27), there is a rerouting of the glycoprotein from its usual site in the myelin to a site of degradation in the lysosomes. This targeting appears to be a mechanism of posttranslational control of the level of P0 in the absence of myelin assembly. Delivery seems to be accomplished by exiting of the glycoprotein after the action of Golgi GlcNAc transferase. This degree of processing, when coupled to the earlier observation that there is a short-lived accumulation of the precursor high-mannose P0 (27), suggests that the species delivered to the lysosomes may form relatively quickly after delivery of the glycoprotein from the ER to the Golgi. Further studies on this mechanism of targeting of the P0 molecule should allow greater understanding of systems used by cells to route proteins to specific sites, and may lead to an understanding of the mechanism of hydrolyase transport in I-cell disease.

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