Expression of the protein zero myelin gene in axon-related Schwann cells is linked to basal lamina formation

Cristina Fernandez-Valle*, Nevis Fregien, Patrick M. Wood, and Mary Bartlett Bunge

The Miami Project to Cure Paralysis and the Departments of Neurological Surgery and Cell Biology and Anatomy, University of Miami School of Medicine, 1600 NW Tenth Avenue, R-48, Miami Florida 33136, USA

*Author for correspondence

SUMMARY

A Schwann cell has the potential to differentiate into either a myelinating or ensheathing cell depending upon signals received from the axon that it contacts. Studies focusing on the pathway leading to myelination demonstrated that Schwann cells must form a basal lamina in order to myelinate an axon. In this report, we describe studies that indicate that initiation of basal lamina synthesis is required for Schwann cells to distinguish between myelination-inducing axons and axons that do not induce myelination, and to respond by undergoing the appropriate genetic and cellular changes. We have used high resolution in situ hybridization, immunocytochemistry and electron microscopy to examine changes in gene expression and morphology of Schwann cells differentiating into myelin-forming cells in vitro. These experiments were carried out in dorsal root ganglion neuron/Schwann cell co-cultures maintained in either serum-free, serum-only or serum-plus-ascorbate-containing medium. We have made four novel observations that contribute significantly to our understanding of how basal lamina and myelination are linked. (1) The addition of ascorbate (in the presence of serum), which promotes basal lamina production, appears to induce expression of the protein zero gene encoding the major structural protein of myelin. Moreover, expression of protein zero mRNA and protein, and its insertion into myelin membranes, occurs only in the subset of Schwann cells contacting myelination-inducing axons. Schwann cells in contact with axons that do not induce myelination, or Schwann cells that have not established a unitary relationship with an axon, do not express protein zero mRNA although they produce basal lamina components. (2) In serum-free conditions, a majority of Schwann cells express protein zero mRNA and protein, but this change in gene expression is not associated with basal lamina formation or with elongation of the Schwann cell along the axon and elaboration of myelin. (3) In the presence of serum (and the absence of ascorbate), Schwann cells again fail to form basal lamina or elongate but no longer express protein zero mRNA or protein. (4) Myelin-associated glycoprotein and galactocerebroside, two additional myelin-specific components, can be expressed by Schwann cells under any of the three culture conditions. Therefore, we have demonstrated that axonal induction of protein zero gene expression in Schwann cells is subject to regulation by both serum- and ascorbate-dependent pathways and that not all myelin-specific proteins are regulated in the same manner. Only when Schwann cells contact axons and initiate basal lamina synthesis is expression of myelin-specific genes restricted to the subset of Schwann cells contacting myelination-inducing axons and coupled to cellular differentiation. In the absence of basal lamina formation, Schwann cells in contact with axons seem to express myelin-specific proteins spuriously without undergoing further differentiation. In sum, these findings suggest that basal lamina serves to simultaneously induce myelin gene expression and cell shape changes in those Schwann cells associated with axons destined for myelination. Basal lamina also suppresses the expression of myelin genes in these SCs in contact with axons that do not induce myelination.

Key words: Schwann cell, myelin-specific gene expression, basal lamina, myelination, protein zero, in situ hybridization, ascorbate

INTRODUCTION

It has long been recognized that axonal properties determine whether Schwann cells (SCs) will myelinate or ensheathe axons. This was definitively shown by cross-anastomosing myelinated with nonmyelinated nerves and observing that the resident SCs of nonmyelinated nerve stumps differentiated into myelinating SCs when interacting with axons originating from myelinated nerves (Weinberg and Spencer, 1975; Aguayo et al., 1976a,b). Both myelinating and non-myelinating (ensheathing) rat SCs arise from a common precursor which is recognized by expression of S100 by embryonic day 15-16 and the O4 antigen (sulfatide) one to two days later (reviewed in Jessen and Mirsky, 1991). SCs...
begin to myelinate large diameter axons soon after birth and then myelinate progressively smaller axons prior to or at the same time as ensheathment of the smallest axons begins, 2-3 weeks after birth (Martin and Webster, 1973; Webster et al., 1973). SCs contacting myelination-inducing axons express myelin-associated glycoprotein (MAG), protein zero (P₀), myelin basic protein, peripheral myelin protein-22 and lipids constituting the myelin sheath including galactocerebrosides (GALC; Politis et al., 1982; Quarles, 1983; Martini and Schachner, 1986; Jessen et al., 1987b; Lemke and Chao, 1988; Mitchell et al., 1990; Snipes et al., 1992). SCs contacting ensheathment-inducing axons (nonmyelinated axons) do not express the myelin proteins but instead synthesize glial fibrillary acidic protein, neural cell adhesion molecule, nerve growth factor receptor and the adhesion molecule, L1, and share with myelinating SCs, the expression of GALC (Jessen et al., 1985; Danilloff et al., 1986; Martini and Schachner, 1986; Mirsky et al., 1986; Jessen et al., 1987b). Loss of axonal contact leads myelinating SCs to stop expressing or to reduce the level of expression of MAG, P₀, myelin basic protein and peripheral myelin protein-22, and to express glial fibrillary acidic protein, neural cell adhesion molecule and nerve growth factor receptor (Politis et al., 1982; Poduslo, 1984; Jessen et al., 1987a; Tanuichi et al., 1988; Trapp et al., 1988; Mitchell et al., 1990; Fan and Gelman, 1992; Snipes et al., 1992). The expression of O4 and GALC are also down-regulated upon loss of axonal contact in both myelinating and ensheathing SCs whereas S100 expression remains constant in both SC types (Jessen et al., 1985; Jessen et al., 1987b). Thus, signals from axons are important determinants of phenotypic-specific gene expression in SCs; these signals, however, have not been identified.

Development of an in vitro myelinating dorsal root ganglion neuron (DRGN)/SC system has allowed detailed studies of cellular mechanisms underlying the complex axon-SC interaction(s) leading to myelination (Wood 1976; reviewed in Wood et al., 1990b). This system has shown that, in addition to axonal contact, basal lamina deposition is an absolute requirement for myelination by SCs (reviewed in Bunge et al., 1990). Deposition of basal lamina is dependent upon the presence of ascorbate in the serum-containing medium nourishing DRGN/SC co-cultures. Ascorbate is required for hydroxylation of proline and lysine residues in type IV collagen (TIVC; Wang et al., 1989) necessary for triple-helical association of collagen chains and leads to increased secretion of triple-helical TIVC (Eldridge et al., 1987; Wang et al., 1989). It has been shown that laminin binding to TIVC requires intact triple-helical TIVC (Woodley et al., 1983) and thus explains the requirement for ascorbate in SC basal lamina assembly, which contains laminin as well as heparan sulfate proteoglycan and entactin (Carey et al., 1983; Cornbrooks et al., 1983; McGarvey et al., 1984; Baron-Van Evercooren et al., 1986; Kuecherer-Ehret et al., 1990). SCs co-cultured with DRGNs in ascorbate-free medium synthesize and secrete only small amounts of non-triple-helical TIVC, which do not interact with other components to form typically assembled basal lamina (Eldridge et al., 1989). Also, SCs contact axons but do not elongate or segregate axons and fail to myelinate. Upon addition of ascorbate to the culture medium, SCs synthesize and secrete triple-helical TIVC, assemble basal lamina and extend along axons. A subset of these SCs forms one-to-one relationships with axons and then engulfs and extends spiralled membranes around them to form multiple myelin lamellae. Myelin internodes appear by the seventh day and myelin abundance increases gradually over the ensuing 2-3 weeks if ascorbate supplementation continues (reviewed in Wood et al., 1990b).

To increase our understanding of the mechanism by which basal lamina deposition is linked to myelination, we asked whether expression of P₀ mRNA encoding the major structural component of peripheral myelin is regulated in individual SCs by conditions that prevent or promote basal lamina and myelin sheath formation. Other investigators have concluded that basal lamina is not involved in inducing myelin gene expression because mRNAs encoding some of the myelin proteins are synthesized by SCs co-cultured with neurons in the absence of basal lamina formation in serum-free culture conditions. These studies, however, did not examine the effect of serum alone on myelin gene expression and were conducted on a mixed population of myelinating and nonmyelinating SCs using biochemical analysis or in situ hybridization techniques that do not afford cellular resolution of mRNAs (Brunden and Brown, 1990; Morrison et al., 1991). We modified our standard DRGN/SC co-culture procedure to permit analysis of gene expression in individual SCs by in situ hybridization. This allowed for the first time a detailed temporal analysis of the sequence of events leading from axonal contact and basal lamina formation to elaboration of myelin sheaths at the single cell level. We were able to show that basal lamina formation (stimulated by the addition of ascorbate) is correlated with the induction of P₀ gene expression in SCs differentiating into myelin-forming cells. Furthermore, our results demonstrate for the first time that basal lamina induces both changes in gene expression and cell shape and restricts expression of myelin genes to the subset of Schwann cells related to myelin-requiring axons.

MATERIALS AND METHODS

DRGN/SC co-cultures

DRGNs of Sprague-Dawley rat embryos at 15 days of gestation (Charles River; Raleigh, NC) were isolated by dissociation with trypsin. Approximately 0.5 dissociated ganglion was plated onto 11 mm glass coverslips coated with poly-L-lysine (Sigma, St Louis, MO) and laminin (Collaborative Research; Bedford, MA) and immersed in Eagle’s Minimum Essential Medium (MEM; Gibco BRL; Gaithersburg, MD), 5% human placental serum and ~50 ng/ml crude nerve growth factor. Cultures were treated with one 3-day pulse of 100 µM fluorodeoxyuridine to eliminate non-neuronal cells and then maintained in MEM plus 5% human placental serum for 4-7 days before seeding with SCs. SCs were isolated from sciatic nerves removed from embryonic day 21 or newborn rat pups and expanded in vitro with forskolin and pituitary extract (Sigma; St Louis, MO) (Brookes et al., 1979). Then 30,000 SCs were added to each DRGN culture and allowed to proliferate in response to axonal mitogens before switching to serum-ascorbate medium [MEM plus 15% fetal bovine serum (FBS), 50 µg/ml ascorbate, and nerve growth factor], serum-only medium (MEM plus 5% or 15% FBS), or serum-free medium (Dulbecco modified Eagle+F12 media), putrescine, sodium...
selenite, progesterone, insulin, transferrin (all components purchased from Sigma, St. Louis, MO) and nerve growth factor; (Bottenstein and Sato, 1979). Increasing the amount of serum from 5 to 15% in the serum-only group or changing the base medium from MEM to DME+FM12 does not alter the results presented here. Details of the tissue culture protocols are provided in Kleitman et al. (1991).

**Preparation of RNA probes**
P₀ cDNA was excised from pSN63 (courtesy of Dr G. Lemke; Lemke and Axel, 1985) and was subcloned into pGEM4Z (Promega; Madison, WI) at the EcoRI site (pGEM4Zp₀). Digoxigenin-labeled antisense and sense RNA probes were synthesized by in vitro transcription using Genius4 (Boehringer Mannheim; Indianapolis, IN). Antisense probes (1.53 kb) were generated by linearizing pGEM4Zp₀ at the ClaI site within the P₀ cDNA and transcribing with T7 polymerase. Sense RNA probes (1.8 kb) were generated by linearizing pGEM4Zp₀ at the SmaI site in the polylinker and transcribing with SP6 polymerase. Samples of sense and antisense P₀ RNA probes and a neomycin control RNA were electrophoresed through formaldehyde agarose gels to verify the sizes, transferred to nitroplus membranes and immunocytochemically detected using an antibody against digoxigenin coupled to alkaline phosphatase (AP). The amount of RNA synthesized was quantitated by dot blotting increasing sample volumes, performing immunocytochemistry and comparing the results with digoxigenin standards provided in the Genius4 kit. RNA probes were then hydrolyzed to approximately 200 nucleotides in length by alkaline hydrolysis and the sizes were again verified as described above.

**In situ hybridization**
Co-cultures were pretreated according to standard protocols (Jordan, 1991) and hybridization and post-hybridization washes were carried out following the procedure of Harland, (1991). Briefly, cultures were rinsed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, exposed to 0.2 N HCl and proteinase K and further fixed in 4% paraformaldehyde. The cells were then acetylated with acetic anhydride, dehydrated with ethanol and prehybridized in 50% formamide, 1×Denhardt's, 4×SSC, 0.05% Tween 20, 0.3% Chaps, 5 mM EDTA and 500 µg/ml each of ssDNA and tRNA overnight at 52°C. Cultures were hybridized for 24 hours at 52°C with 1 µg/ml digoxigenin-labeled antisense or sense P₀ RNA probe in prehybridization buffer. Post-hybridization washes entailed gradually exchanging hybridization buffer for 2× SSC plus 0.3% Chaps, incubating cultures with RNase A and T1 and carrying out several high-stringency washes with 0.2× SSC plus 0.3% Chaps at 52°C for 30 minutes each. Several more rinses were carried out to gradually exchange 0.3% Chaps for 0.1% Tween in PBS.

**Immunocytochemical detection of RNA hybrids and P₀ protein**
P₀ RNA-RNA hybrids and P₀ protein were detected as follows: After post-hybridization washes were completed, cultures were rinsed in PBS with 2 mg/ml bovine serum albumin (BSA) and 0.1% Triton X-100 and were incubated overnight at 4°C with 1:3000 dilution of anti-digoxigenin sheep polyclonal antibody conjugated to AP (Boehringer Mannheim) and a 1:333 dilution of anti-P₀ rabbit polyclonal antibody (kindly provided by Dr D. Colman) in PBS, BSA, 0.1% Triton X-100 and 20% goat serum. Cultures were rinsed extensively in PBS with BSA, Triton X-100, and 20% goat serum (3-5 changes over 2 hours). A 1/100 dilution of fluorescein goat anti-rabbit antibody in PBS with BSA, Triton X-100 and 20% goat serum was added, and cultures were incubated for 45 minutes at room temperature. Cultures were rinsed extensively with the same buffer (3-5 times/hour) and then endogenous AP activity was inhibited by a 10 minute incubation with 5 mM levamisole (Sigma; St. Louis, MO) in 100 mM Tris HCl, pH 9.5, 50 mM MgCl₂, 100 mM NaCl and 0.1% Tween 20. To produce the color precipitate, cultures were incubated in a solution containing nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂ and 100 mM NaCl for 3-5 hours at room temperature. To stop the AP reaction, cultures were rinsed in 10 mM Tris-HCl, pH 8, with 1 mM EDTA. Cultures were additionally fixed in 4% paraformaldehyde to preserve the reaction product, mounted in a glycerol/PBS solution containing an anti-bleaching agent (Citifluor; Canterbury, England), and viewed by differential interference-contrast microscopy and fluorescence microscopy on a Zeiss axiophot microscope.

**Immunocytochemistry**
Co-cultures not used for in situ hybridization were immunostained for GALC, MAG and TIVC using standard protocols. Briefly, DRGN/SC co-cultures were rinsed in PBS several times and non-specific binding was blocked by preincubation with PBS containing 10% goat serum. GALC and MAG immunostaining were carried out using undiluted 01 hybridoma medium and concentrated S13 hybridoma medium, respectively (01 and S13-secreting cells kindly provided by Dr M. Schachner). TIVC immunostaining was carried out with a 1:200 dilution of a polyclonal antibody to the non-collagenous domain of TIVC (kindly provided by Dr Charonis). Incubation with primary antibodies was done for 30 minutes at room temperature and was followed by several rinses in PBS/goat serum. A 1:100 dilution of either rhodamine or fluorescein goat anti-mouse or goat anti-rabbit was added and cultures were incubated for an additional 30 minutes at room temperature. Co-cultures were rinsed in PBS, fixed in 4% paraformaldehyde, mounted in Citifluor and were viewed on a Zeiss universal microscope equipped with fluorescence optics.

**Electron microscopy**
DRGN/SC co-cultures were fixed in buffered glutaraldehyde followed by osmium tetroxide, dehydrated in ethanol and embedded in EMBed (EMS; Fort Washington, PA). Areas to be examined were selected, sectioned at 1 µm and stained (with toluidine blue) for light microscopy, and thin sectioned for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate and viewed in a Philips 300 electron microscope.

**Cell counts**
To determine the number of SCs expressing P₀ mRNA in the time course experiments, entire 11 mm coverslips were scanned manually under differential interference-contrast optics at 200 times magnification. For long-term cultures (3 weeks in myelination medium), an approximate estimate of positive cells was made based on the number of SCs seeded initially, the number of SCs known to provide a confluent, dense layer of SCs on an 11 mm coverslip and the approximate area covered by the alkaline phosphatase reaction product.

**RESULTS**

**P₀ mRNA is detected in myelinating but not in nonmyelinating SCs co-cultured with DRG neurons**
Digoxigenin-labeled RNA probes were used to detect P₀ mRNA in situ hybridization in individual SCs co-cultured with DRGNeurons. RNA-RNA hybrids were detected by indirect immunocytochemistry using a polyclonal antibody to digoxigenin, coupled to alkaline phosphatase (AP). The intracellular color precipitate produced by the AP reaction provides greater cellular resolution than that obtained by
P₀ mRNA expression is linked to basal lamina deposition

In situ hybridization analysis was conducted on DRGN/SC co-cultures maintained for 3 weeks in serum-containing medium with or without ascorbate supplementation. In four co-cultures receiving ascorbate to permit basal lamina deposition, approximately one third of the 60,000 SCs in each co-culture expressed P₀ mRNA (Fig. 2A). In contrast, when basal lamina deposition was prevented by withholding ascorbate from the serum-containing medium, none of the SCs in three sibling co-cultures and only 40 out of 60,000 SCs in a fourth co-culture expressed P₀ mRNA (Fig. 2B).

This result was surprising in view of previous work done by others demonstrating that P₀ was expressed by SCs co-cultured with neurons in serum-free medium (Brunden and Brown, 1990; Morrison et al., 1991). This suggests that serum suppressed axonal induction of P₀ gene expression. This interpretation is discussed further below. These results further indicate that P₀ expression is regulated at the transcriptional or post-transcriptional level rather than at the translational or post-translational level by a mechanism linked to basal lamina assembly in SCs differentiating into myelin-forming cells.

Expression of P₀ mRNA and protein coincides with morphological differentiation

A time course study of the induction of P₀ gene expression was conducted in DRGN/SC co-cultures during the first week following addition of ascorbate to the serum-containing medium. The earliest time P₀ mRNA was detected was 3 days after ascorbate addition; however, this was restricted to only a few SCs (2-4 SCs/culture) expressing very low levels of P₀ mRNA (Fig. 3A). The P₀ mRNA was translated into protein detectable by immunostaining (Fig. 3B); this immunostaining was useful in determining the extent of SC elongation along the axon. These SCs were morphologically undifferentiated; i.e., they had extended only short cytoplasmic processes along the axons and had rounded nuclear profiles. By 5 days, some SCs (~150 SCs/culture) expressed very high levels of P₀ mRNA (Fig. 3C). The intensity of the AP reaction product was so great that it obscured immunostaining for P₀ protein in the perinuclear area (Fig. 3D). SCs displayed characteristics associated with an intermediate state of differentiation including cellular and nuclear elongation and P₀ immunostaining.
Fig. 3. Expression of $P_0$ mRNA and protein coincides with cellular shape changes indicative of differentiation into myelin-forming cells. (A) At 3 days after serum+ascorbate addition, only a few SCs express $P_0$ mRNA at very low levels (arrowhead, SC nucleus). (B) $P_0$ mRNA is translated into protein, as shown in the corresponding immunostained image. Although the SC nucleus remains rounded, the SC has begun to elongate and distribute $P_0$ protein along its length (arrowhead, SC nucleus). (C) At 5 days after serum+ascorbate addition, some SCs contain abundant levels of $P_0$ mRNA as noted by the AP reaction product filling the perinuclear area (arrowhead, SC nucleus). (D) $P_0$ protein is located along the extent of the SC cytoplasm; it is obscured in the perinuclear area (arrowhead) by the histochemical reaction product. (E) At 6 days after serum+ascorbate addition, many SCs express varying amounts of $P_0$ mRNA. The pair of arrows delineates the extent of SC elongation in one case. (F) The distribution of $P_0$ protein is shown in the corresponding immunostained image. $P_0$ immunostaining enables detection of the elongating SC, such as is found between the arrows. (G) At 8 days after serum+ascorbate addition, $P_0$ mRNA abundance is decreased in mature SCs possessing flattened and elongated nuclei (arrowhead). (H) The corresponding immunostained image shows $P_0$ distributed along internodes separated by typically spaced nodes of Ranvier marked by arrows. $P_0$ immunostaining is no longer obscured over the SC nuclear area (arrowhead) because $P_0$ mRNA abundance has decreased. Scale bar, 10 µm.
visible throughout the cell. At 6 days, hundreds of SCs expressed varying levels of P₀ mRNA (Fig. 3E) and appeared to be establishing their axonal domains. Most of the SCs contained elongated nuclei and P₀ protein was distributed along the extent of the SC cytoplasm (Fig. 3F). By the 8th day following ascorbate addition, approximately 5-6000 SCs/co-culture expressed P₀ mRNA. The most mature SCs formed myelin internodes separated by nodes of Ranvier and contained flattened and elongated nuclei; these SCs displayed a decreased level of P₀ mRNA expression (Fig. 3G). At this stage, compact myelin sheaths could be readily visualized by their typical refraction under bright-field microscopy (Bunge et al., 1989). P₀ protein was distributed along the entire internode as well as in the perinuclear area of the SC (Fig. 3H). At 21 days after ascorbate addition, the number of SCs expressing P₀ mRNA had continued to increase (Fig. 2A), but the abundance of P₀ mRNA/SC varied in relation to the SC’s state of differentiation which in turn varied due to the asynchronous onset of myelination. These results demonstrate that the onset of P₀ expression coincides with the initiation of morphological differentiation in SCs and suggest that P₀ may play a role in the early events of myelin formation in addition to its role in compaction of myelin membranes.

**Initiation of basal lamina deposition coincides temporally with P₀ mRNA expression**

Co-cultures prepared in the same manner as those used for in situ hybridization analysis were studied ultrastructurally to determine the extent of basal lamina deposition and SC differentiation at the time P₀ mRNA was first expressed at high levels. SCs in co-culture with DRGNs for only 3 days in the presence of serum+ascorbate exhibited very irregular surfaces due to numerous membranous protrusions (Fig. 4A). This was suggestive of membrane or cytoskeletal reorganization as the SC developed plasmalemmal domains that were either axobinal (away from the axon) or adaxonal (adjacent to the axon). Very little basal lamina was present at this time although some SCs were beginning to elongate along axons. In contrast, by 5 days of co-culture with axons in serum+ascorbate, some SCs displayed smooth plasmalemmal membranes and had deposited substantial lengths of basal lamina (Fig. 4B,C). The most differentiated SCs at this time had elongated along (Fig. 4B) and completely engulfed (Fig. 4C) an axon and had formed a mesaxon; in some SCs the mesaxon had spiralled up to 1.5 turns around an axon. Basal lamina deposition was becoming continuous on these most differentiated SCs. SCs cultured in serum-only medium (lacking ascorbate) did not form basal lamina and displayed smooth plasma membranes and round nuclear profiles. Although some SCs contacted and partially surrounded some of the large diameter axons (Fig. 4D), they did not form a mesaxon, a step that precedes spiral formation. These results indicate that the onset of basal lamina deposition occurs at the same time as the onset of P₀ mRNA expression and elongation of the SC along an axon. The formation and spiralling of the mesaxon around an axon to form multiple membranes occurs at the time when P₀ mRNA levels are highest in the differentiating SCs.

**SCs co-cultured with axons in serum- and ascorbate-free medium express P₀ mRNA and protein but do not elongate or myelinate**

Because of previous reports that SCs expressed low levels of P₀ mRNA when co-cultured with sensory neurons in serum-free medium (Brunden and Brown, 1990; Morrison et al., 1991), we carried out in situ hybridization analysis of DRGN/SC co-cultures maintained under these conditions. Our results confirmed that many SCs expressed low levels of P₀ mRNA and protein when co-cultured with neurons in serum- and ascorbate-free medium (Fig. 5A,B), indicating that axonal contact alone induces P₀ gene expression in most SCs in serum-free culture conditions. These P₀-expressing SCs did not form myelin sheaths and, moreover, remained morphologically immature as defined by round nuclear profiles (Fig. 5C) and short cytoplasmic extensions visualized by immunostaining. This morphology resembles that of SCs co-cultured in serum-only medium (Fig. 5D) rather than that of SCs co-cultured in serum+ascorbate where they exhibit nuclear and cellular elongation (Fig. 5E).

To characterize further this axonally mediated and basal lamina independent expression of P₀ mRNA in serum-free medium, DRGN/SC co-cultures maintained in serum-free, serum-only or serum+ascorbate medium for 2 to 8 days were compared. At 3 days after addition of the respective media, we found that the number of SCs expressing P₀ mRNA and protein was greater in co-cultures maintained in serum-free medium than those maintained in serum+ascorbate medium. The number of SCs expressing P₀ mRNA in serum-free medium increased thereafter up to 8 days, the longest time examined. The early induction of P₀ expression in serum-free medium by SCs unable to myelinate suggests that the mechanism by which this occurs differs from that operating in SCs maintained under basal lamina and myelin permissive conditions. To determine whether this expression of P₀ affected the typical pattern or time course for myelination, some co-cultures were grown for 5 days in serum-free medium and then switched to serum+ascorbate medium for an additional 3 days. Whereas most SCs co-cultured in serum-free medium for 5 days expressed P₀ but did not differentiate (Fig. 5F), the majority of the SCs no longer expressed P₀ mRNA when switched to serum+ascorbate medium. A minority of the SCs (~1200 SCs/co-culture), however, continued to express P₀ mRNA and protein. These SCs were elongated and displayed morphological characteristics indicative of myelination after just 3 days in serum+ascorbate medium (Fig. 5G). Prior exposure to serum-free medium that induced P₀ expression in SCs appeared to accelerate differentiation of a subset of SCs into myelin-forming cells; presumably these SCs were those contacting myelination-inducing axons. Normally, SCs require 7 days in serum+ascorbate medium to reach an equivalent level of differentiation. Some co-cultures grown in serum-free medium for 5 days were subsequently switched to serum-only medium for 3 days. The SCs in these co-cultures no longer expressed P₀ mRNA (Fig. 5G), demonstrating that serum suppresses P₀ expression in SCs and confirming that the addition of ascorbate to the serum-containing medium leads to induction of P₀ mRNA expression in a subset of SCs. Additionally, these results
Fig. 4
Ascorbate induces P₀ gene expression

indicate that, although an axon-dependent mechanism exists in SCs for induction of the P₀ gene, this mechanism is subject to further regulation by a factor(s) present in serum and by ascorbate (which increases synthesis and secretion of TIVC necessary for basal lamina assembly). Precocious induction of P₀ expression in serum-free conditions primes
the SC to progress to myelination more rapidly when permissive conditions are provided.

Expression of other myelin-related molecules by SCs unable to form basal lamina or myelin
SCs co-cultured with neurons in the presence or absence of serum, with or without ascorbate, were immunostained with antibodies to the myelin components, GALC and MAG, to determine whether these molecules were co-expressed with P₀ when basal lamina and myelin formation were prevented. Immunostaining for the basal lamina component, TIVC, was also performed to indicate the extent of extracellular matrix deposition. We found that GALC and MAG were expressed in SCs 5 days after the addition of serum-only (Fig. 6D,E) or serum-free (Fig. 6G,H) medium. The staining pattern of GALC on SCs in serum-only medium (Fig. 6D) closely resembled the intensity of staining seen on SCs co-cultured in myelin-permissive medium (Fig. 6A) except that SCs were not as elongated. MAG immunostaining on SCs co-cultured in serum-only medium (Fig. 6E) was not as intense as that found on sibling SCs co-cultured in serum+ascorbate (Fig. 6B). At the time points shown, MAG and GALC

Fig. 6. Expression of MAG, GALC and TIVC in co-cultures maintained in serum-free, serum-only or serum+ascorbate medium. (A-C) Co-cultures in serum+ascorbate medium; (D-F) serum-only medium; and (G-H) serum-free medium. (A) Immunostaining for GALC at 4 days and (D,G) 6 days, or (B,E,H) MAG at 6 days. Varying levels of both myelin components are expressed by SCs co-cultured with neurons in the presence or absence of serum and/or ascorbate. (C) Immunostaining for TIVC on SCs after 4 days in serum+ascorbate medium and (F) 6 days in serum-only or (I) serum-free medium. (C) Within 4 days of ascorbate addition, SCs display a linear, blanket-like staining for TIVC, whereas SCs co-cultured with neurons in serum-only (F) or serum-free media (I) display dim and punctate staining for TIVC. This degree of TIVC secretion is required for SCs to deposit basal lamina and to myelinate axons. Scale bar, 10 μm.
expression is lower in the serum-free and serum-only conditions than in the serum+ascorbate condition. Upon continued culture (2-3 weeks), both the number of SCs expressing MAG and GALC as well as the intensity of the immunostaining increases substantially in serum-free and serum-only conditions (Wood et al., 1990b). Expression of GALC and MAG by SCs in serum-only or serum-free medium occurred in the absence of substantial TIVC deposition by the SCs. Only very low levels of TIVC were detected on SCs 4-6 days after addition of either serum-only or serum-free medium (Fig. 6F,I). The staining pattern was punctate and did not resemble the linear immunostaining seen on SCs co-cultured in serum+ascorbate medium (Fig. 6C). TIVC immunoreactivity on SCs quickly advanced from a dim and punctate pattern to the mature pattern in less than 4 days after the first ascorbate addition (Fig. 6C). GALC and MAG expression was not suppressed in the presence of serum, indicating a different regulatory mechanism from that for P0.

**DISCUSSION**

**P0 mRNA expression in SCs is linked to basal lamina assembly**

SCs co-cultured with neurons in serum-free medium respond to axonal signals and express P0 mRNA but are unable to elongate or form myelin because basal lamina is not formed. SCs co-cultured with neurons in serum-only medium no longer express P0 mRNA, do not form basal lamina or myelin and remain morphologically undifferentiated. Whereas P0 mRNA is not found in these SCs, other myelin-specific molecules, GALC and MAG, are expressed. A subset of SCs co-cultured with neurons in the presence of serum+ascorbate forms basal lamina, expresses P0 mRNA and P0 protein, elongates, engulfs axons and assembles P0 into myelin sheaths. The onset of P0 mRNA expression, basal lamina deposition and SC elongation all commence at about the same time, i.e. 3-5 days after the first ascorbate addition. These observations demonstrate for the first time that expression of the P0 gene is linked to basal lamina assembly.

This finding is in opposition to those made earlier by other investigators (Brunden et al., 1990; Brunden and Brown, 1990; Morrison et al., 1991). They observed that SCs co-cultured with DRGNs in serum- and ascorbate-free medium (which does not permit basal lamina deposition) express both P0 mRNA and protein and concluded that basal lamina formation was therefore not needed for myelin gene expression. This expression is presumably in response to axonal contact since Schwann cells cultured without axons do not express myelin genes unless stimulated with forskolin to elevate cAMP levels (Lemke and Chao, 1988; Morgan et al., 1991). We noted that Schwann cells expressing myelin genes in serum-free cultures did not show other signs of differentiation and that P0 gene expression was abolished by serum. Only when both serum and ascorbate are added to permit basal lamina deposition, do those SCs associated with axons requiring myelination express P0. All other Schwann cells cease to express any myelin genes. This observation expands the already well known requirement of basal lamina for myelination. It tells us that basal lamina is required for induction of myelin genes and that it serves to specify myelin formation by allowing Schwann cells to recognize and/or respond to axonal signals that determine the final differentiated state of the SC. Thus, these results suggest a new function for basal lamina.

Earlier findings showed that in long-term cultures many more SCs express GALC and MAG when co-cultured in serum-free medium than when co-cultured in serum+ascorbate medium (Wood et al., 1990b). In serum-free conditions, expression of GALC and MAG is not dependent upon contact with axons destined for myelination whereas, in the presence of serum+ascorbate, expression of myelin components is coupled with myelination and restricted to SCs contacting myelin-inducing axons. Basal lamina formation therefore appears to induce P0 gene expression in SCs contacting axons destined for myelination and to suppress MAG and GALC expression by SCs associated with nonmyelinated axons, thereby restricting expression of myelin components (by apparently different mechanisms) to SCs engaged in myelination. Basal lamina formation is also required for changes in SC shape underlying engulfment of and spiralization around an axon. In serum-free medium, expression of the myelin components P0, GALC and MAG is induced in SCs but cellular shape changes are not and myelination fails to occur. Further, this observation indicates that the pathways leading to myelination gene activation and cell shape change can be independently activated.

Indeed, as mentioned earlier, P0 gene expression can be induced in isolated SCs in the absence of neuronal contact by agents that increase intracellular cAMP levels (Lemke and Chao, 1988; Morgan et al., 1991). cAMP does not directly induce P0 gene expression because cAMP response elements have not been found in the promoter region of the P0 gene (Lemke et al., 1988). It is not known whether cAMP levels increase in axon-related SCs forming basal lamina in vitro and how this would lead to myelin gene expression. P0 gene expression can be repressed in proliferating SCs by the POU transcription factor, SCIP. This “suppressed cAMP inducible POU” protein is transiently expressed in SCs only during times of rapid proliferation that occur during development and following loss of axonal contact (Monuki et al., 1989, 1990). It is not known if SCIP is expressed in SCs co-cultured with neurons in vitro and if elevated SCIP levels may explain the observed suppression of P0 gene expression in SCs co-cultured with neurons in serum-only medium.

Basal lamina synthesis appears to be necessary for SCs to discriminate between myelination-inducing and ensheathment-inducing axons and to express myelin components appropriately. Results of the time course and correlative ultrastructural studies demonstrate that P0 mRNA expression and SC elongation along an axon begin before a continuous basal lamina is deposited. Therefore, only initiation of basal lamina assembly and not the deposition of a fully formed basal lamina is required for induction of P0 gene expression and SC differentiation. The possibility that ascorbate directly stimulates P0 gene expression exists since ascorbate is known to increase transcription rates for pro-collagen α2 (I) mRNAs in chick tendon (Lyons and Schwarz, 1984) and steady state levels of mRNAs encoding...
type I collagen in human fibroblasts (Chojkier, et al., 1989; Kurata and Hata, 1991; Phillips et al., 1992). However, the time between addition of ascorbate and appearance of P0 mRNAs (3-5 days) is longer than the time between ascorbate addition and induction of collagen gene expression, which is detectable by 20 hours and is maximal at 60-72 hours (Lyons and Schwarz, 1984; Chojkier et al., 1989). On this basis, it appears unlikely that ascorbate induces P0 expression directly. Another perhaps more convincing argument for the ascorbate effect to be mediated by basal lamina formation is the observation that oligodendrocytes, which do not form basal lamina, do not require ascorbate for expression of myelin genes and elaboration of myelin sheaths in vitro (Eldridge et al., 1989). Therefore, whereas it is not possible at this time to rule out the possibility that ascorbate leads to P0 gene expression via a mechanism independent of basal lamina formation, our present findings are consistent with the hypothesis that binding of basal lamina components to the SC surface initiates a pathway leading to myelin formation.

Basal lamina assembly is not a guarantee, however, that SCs will myelinate. SCs must also interact with the L1 adhesion molecule (and possibly other molecules) expressed on the surface of myelination-inducing axons in order to form a myelin sheath. In the presence of L1 antibodies, SCs deposit some basal lamina but fail to elongate along axons and to express the myelin-specific molecules, GALC and MAG (Seilheimer et al., 1989; Wood et al., 1990a). A functional cytoskeleton is another requirement for myelination. In the presence of cytochalasin D, which disrupts actin filaments, SCs deposit a rudimentary basal lamina but again fail to myelinate (Gorman and Bunge, 1988). These observations demonstrate that SCs require the coordinated activation of myelin-gene expression and the cytoskeleton with basal lamina assembly and interaction with axonal molecules to elaborate a myelin sheath.

How are basal lamina assembly and myelination linked?

An hypothesis consistent with these observations is that, when SCs contact axons in the presence of serum-ascorbate, they begin to assemble extracellular matrix components into an immature basal lamina prefatory to establishing plasmamembranous domains (Bunge et al., 1983, 1986). This allows polarization of the SC so that one domain away from the axon (abaxonal) becomes covered by basal lamina whereas the other domain adjacent to the axon (adaxonal) is able to more extensively interact with axonal surface components. Polarization is required for the reorganization of both surface and cytosolic domains within the SC; this reorganization leads to cellular shape changes such as elongation and spiralling. As preexisting or newly synthesized surface molecules reach the adaxonal domain, they interact with axonal signals (such as L1). The axonal signals recognized by the SC during this interaction determine whether a SC will differentiate into a myelinating or ensheathing SC. In myelinating SCs, genes encoding neural-cell adhesion molecule and nerve growth factor receptor (for example) are down-regulated while those encoding P0 and myelin basic protein are up-regulated. The activation of the cytoskeleton and increased membrane synthesis allows the SC to elongate, engulf, and spiral around the axon. Since all axons, myelinated and ensheathed, express L1, this molecule is not likely to signal myelination itself but rather is required for myelin formation. Only a subset of axons express the molecule(s) inducing SC myelination. Alternatively, all axons express this molecule but in amounts proportional to their surface area. This has been suggested by others to account for the observation that myelination is largely dependent upon axon diameter (Friede, 1972; Webster and Favilla, 1984; Voyvodic, 1989). When SCs encounter a threshold level of this molecule, myelination commences.

It is clear that elaboration of a myelin sheath requires more than just expression of myelin-specific genes. Myelination requires coordination of myelin gene expression with cellular events that increase lipid synthesis and activate cytoskeletal reorganization that promotes SC movement along and around an axon. A functional actin network is required for myelination (see above), but little is known about the SC receptors for basal lamina components such as laminin and how they interact with the cytoskeleton in SCs. Integrins are good candidates for study since they participate in bridging the extracellular matrix with the cytoskeleton (Mueller et al., 1989; Marchisio et al., 1991). The DRGN/SC co-culture system allows the application of molecular and cellular techniques to examine the mechanisms underlying differential gene expression and cell shape changes in individual SCs at various stages of differentiation into a myelin-forming cell.

This work was supported by NIH 5F32NS09006 (CFV), NINDS NS09923 (R.P.Bunge), the National Multiple Sclerosis Society RG2210-A-2 (P. M. W.), and The Miami Project to Cure Paralysis. We thank the following for their generous gifts: Dr Lemke for the pSN63 plasmid containing a P0 cDNA, Dr Colman for P0 antibody, Dr Schachner for MAG and GALC antibody-producing cell lines and Dr Charonis for TIVC antibody. We also thank L. Hurst, A. Gomez, M. Bates and F. Cruz for technical assistance and R. Camarena for photographic work. We are grateful to DRS. Nestor Barrezeuta and Scott Stachel for helpful discussions regarding the in situ hybridization technique.

REFERENCES


Ascorbate induces P0 gene expression

879


(Accepted 26 July 1993)