Axonal Regulation of Schwann Cell Integrin Expression Suggests a Role for α6β4 in Myelination

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Abstract. Ensheathment and myelination of axons by Schwann cells in the peripheral nervous system requires contact with a basal lamina. The molecular mechanism(s) by which the basal lamina promotes myelination is not known but is likely to reflect the activity of integrins expressed by Schwann cells. To initiate studies on the role of integrins during myelination, we characterized the expression of two integrin subunits, β1 and β4, in an in vitro myelination system and compared their expression to that of the glial adhesion molecule, the myelin-associated glycoprotein (MAG). In the absence of neurons, Schwann cells express significant levels of β1 but virtually no β4 or MAG. When Schwann cells are cocultured with dorsal root ganglia neurons under conditions promoting myelination, expression of β4 and MAG increased dramatically in myelinating cells, whereas β1 levels remained essentially unchanged. (In general agreement with these findings, during peripheral nerve development in vivo, β4 levels also increase during the period of my-

elination in sharp contrast to β1 levels which show a striking decrease.) In cocultures of neurons and Schwann cells, β4 and MAG appear to colocalize in nascent myelin sheaths but have distinct distributions in mature sheaths, with β4 concentrated in the outer plasma membrane of the Schwann cell and MAG localized to the inner (periaxonal) membrane. Surprisingly, β4 is also present at high levels with MAG in Schmidt-Lanterman incisures. Immunoprecipitation studies demonstrated that primary Schwann cells express β1 in association with the α1 and α6 subunits, while myelinating Schwann cells express α6β4 and possibly α1β1. β4 is also downregulated during Wallerian degeneration in vitro, indicating that its expression requires continuous Schwann cell contact with the axon. These results indicate that axonal contact induces the expression of β4 during Schwann cell myelination and suggest that α6β4 is an important mediator of the interactions of myelinating Schwann cells with the basal lamina.

D URING development, Schwann cells ensheathe or myelinate nerve fibers in the continuous presence of basal lamina (Webster et al., 1973). The basal lamina is deposited on the outer surface of the Schwann cell–axon unit and is required for normal ensheathment or myelination of axons (Bunge and Bunge, 1986). This requirement has been most clearly demonstrated by in vitro studies in which purified populations of Schwann cells were cocultured with neurons under conditions that either promote or inhibit the formation of the basal lamina. Such studies have demonstrated that Schwann cells do not assemble a basal lamina when cultured in media lacking ascorbic acid, which is needed for the formation of triple-helical collagen (Eldridge et al., 1987), or in the presence of cis-4-hydroxy-L-proline, a biosynthetic inhibitor of collagen for-

mation (Eldridge et al., 1988). Under such conditions Schwann cells fail to ensheathe or myelinate neurites normally. Reversal of the basal lamina defect in these cultures by adding ascorbic acid or removing the biosynthetic inhibitor respectively, or by adding exogenous extracellular matrix materials (Carey et al., 1986), resulted in a rapid correction of the ensheathment defect and formation of normal myelin sheaths.

Additional evidence supporting a requirement for the basal lamina during Schwann cell myelination has come from studies of the dystrophic mouse mutant. In this spontaneously occurring mouse mutant, defects in Schwann cell ensheathment and myelination are present in the proximal spinal roots (Bradley and Jenkison, 1973; Stirling, 1975) and have been correlated with associated defects in the Schwann cell basal lamina (Madrid et al., 1975). While the precise molecular defect in these animals is not known, these studies provide correlative support for the importance of the basal lamina for Schwann cell function.

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Although these findings indicate that interactions between the Schwann cell and the extracellular matrix are critical for Schwann cell ensheathment and myelination, little is known about how the basal lamina regulates Schwann cell function. Despite the failure of Schwann cells to ensheathe or myelinate in the absence of a basal lamina, axons are still able to induce the proliferation of Schwann cells (Moya et al., 1980) and their expression of several myelin-related components, notably galactose cerebroside, the myelin-associated glycoprotein (MAG),[1] and P0 (Owens and Bunge, 1989; Brunden and Brown, 1990). Interestingly, in the absence of a basal lamina, MAG, which is normally present in the inner turn of the myelin sheath where it is believed to promote adhesion to the axon (Trapp and Quarles, 1982), is instead diffusely present at the Schwann cell surface, not just in proximity to the axon (Owens and Bunge, 1989). These results suggest that, while the basal lamina is not required for myelin protein synthesis, it is required for assembly of the myelin sheath which, in turn, may depend on the ability of the Schwann cell to establish an appropriate cellular polarity, with adaxonal and abaxonal surfaces (Bunge et al., 1986). In addition, the presence of the basal lamina could provide critical mechanical support to the Schwann cell as it extends processes around the axon during ensheathment or myelination. This is consistent with observations that the outer surface of the Schwann cell, which is in contact with the basal lamina, remains essentially stationary whereas the inner loop progressively circumnavigates around the axon during myelination (Bunge et al., 1989). It is also possible that, as in other systems, the extracellular matrix may have a role in transmembrane signaling of Schwann cells via extracellular matrix receptors (Hynes, 1992; Juliano and Haskill, 1993).

The requirement for a basal lamina suggests that integrins have a critical role in Schwann cell function. Integrins are heterodimeric receptors, consisting of an α and β subunit, that mediate the interactions of cells with extracellular matrices and other cells (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Reichardt and Tomaselli, 1991). Limited studies have been done to date on the integrins expressed by Schwann cells. These studies suggest that the integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$, and $\alpha 6\beta 4$ are expressed by some Schwann cells in peripheral nerves (Sonnenberg et al., 1990; Toyota et al., 1990; Hsiao et al., 1991; Lefcort et al., 1992; Jaakkola et al., 1993). However, very little is known about the regulation of their expression during Schwann cell differentiation, their distribution in peripheral nerve, or their function during myelination.

To initiate studies on the mechanisms by which the basal lamina regulates Schwann cell myelination, and the role of integrins in mediating these interactions, we have characterized, in an in vitro myelination system, the expression of the $\beta 1$ and $\beta 4$ integrins. Culturing Schwann cells alone or with neurons has allowed us to investigate the role of the axon in regulating Schwann cell integrin expression in the absence of other factors, including perineurial cells, that potentially complicate similar analyses in vivo. We have found that

the $\alpha 6\beta 1$ laminin receptor is constitutively expressed by Schwann cells, even in the absence of axonal contact, whereas expression by Schwann cells of another basement membrane receptor, $\alpha 6\beta 4$, is axonally regulated and dramatically increases at the onset of myelination in vitro. Immunolocalization studies suggest that $\alpha 6\beta 4$ may be present in the uncompacted membranes of the nascent myelin sheath, whereas in mature myelin, it is expressed at the outer Schwann cell membrane and in some Schmidt-Lanterman clefts. These results suggest that $\alpha 6\beta 4$ is likely to have an important role in promoting Schwann cell myelination via its interactions with the basal lamina.

Materials and Methods

Antibodies

The $\alpha 6$, $\beta 4$, and $\beta 1$ subunit-specific rabbit polyclonal antibodies were generated against synthetic peptides corresponding to the carboxy-terminal sequences of the human integrin subunits and have previously been described (Giancotti and Ruoslahti, 1990; Giancotti et al., 1992). The $\alpha 6$ antibody was generated against a peptide sequence which is exclusively present in the alternatively spliced cytoplasmic variant of $\alpha 6$, referred to as $\alpha 6A$ (Hogervorst et al., 1993). In all cases, affinity-purified antibodies were used except for the immunoprecipitation studies in which total serum was used. The anti-MAG mAb MA513, was a kind gift of M. Shachner (Swiss Federal Institute of Technology, Zürich). A rabbit polyclonal antibody, which was generated against the extracellular domain of rat MAG as previously described (Pedraza et al., 1991), was also used in some experiments. A rabbit polyclonal antibody to myelin basic protein (MBP) was a kind gift of D. Colman (Mt. Sinai Medical Center, New York, NY). Nuclei labeled with bromodeoxyuridine (BrDU) were detected with fluorescein-conjugated mouse mAbs directed against BrDU (Boehringer Mannheim Corp., Indianapolis, IN).

Tissue Culture Methods

Rat primary Schwann cells were prepared by the method of Brockes (Brockes et al., 1979) as modified by Porter et al. (Porter et al., 1986). Primary Schwann cells were routinely maintained on a standard media consisting of MEM (Whittaker Bioproducts, Inc., Walkersville, MD) supplemented with 10% FBS, 2 mM glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF (Bioproducts for Science, Inc., Indianapolis, IN). In some studies, confluent cultures of Schwann cells were continuously treated for 2 wk with a crude preparation of glial growth factor (GGF) and 2 μM forskolin, which were added as supplements to the standard media as previously described (Porter et al., 1986).

Schwann cell/neuron cultures were established with minor modifications of previously described methods (Kleitman et al., 1991). Briefly, dorsal root ganglia (DRG) were removed from E15 or E16 rats, dissociated with trypsin, and plated onto 13 mm glass coverslips coated with ammoniated rat tail collagen (Biomedical Technologies, Inc., Stoughton, MA). Cultures were treated for 2 wk with 5-fluorodeoxyuridine and uridine (both at 10^{-5} M) which were added to the standard media in alternate feedings to eliminate nonneuronal cells. Purified neurons were seeded with rat primary Schwann cells (100,000–200,000 Schwann cells/coverslip). On the following day the standard media was replaced with the defined media, N2 (5 mg/ml insulin, 10 mg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM selenium, 2 mM glutamine in a 1:1 mixture of DME and Ham's F-12 supplemented with 2.5S NGF). In N2 media, Schwann cells are unable to assemble a basal lamina or myelinate but continue to proliferate in association with neurites (Moya et al., 1980). The cultures were maintained on N2 for several days until the neurites were fully populated with Schwann cells. They were then fed the standard media supplemented with 50 μg/ml ascorbic acid in order to promote basal lamina formation and initiate myelination.

To examine the expression of $\beta 4$ in myelinating Schwann cells during Wallerian degeneration, undissociated DRG explants from E16 rats were plated in pairs (a total of four ganglia) on opposite edges of collagen-coated coverslips (20 mm in diameter) fashioned from Aclar plastic. After treatment with antimitotic agents, approximately 250,000 Schwann cells were added per coverslip. The cocultures were initially maintained on defined

media and then switched to myelination promoting media for one month. To initiate Wallerian degeneration, ganglia on one side of the coverslip were excised with a razor blade and removed; myelinating ganglia on the other side of the coverslips served as controls. One day after axotomy the standard culture media containing ascorbic acid was supplemented with BrDU (Sigma Immunochemicals, St. Louis, MO) to a final concentration of 4 nM for a total of 3 d. Cultures were photographed prior to and immediately after axotomy to facilitate subsequent identification of the degenerating myelin sheaths.

Immunofluorescence Microscopy

Cultures grown on coverslips were rinsed in PBS (dPBS; Gibco BRL, Gaithersburg, MD), fixed with 3.7% formaldehyde in PBS for 10 min, washed in PBS, and then permeabilized with 100% methanol at −20°C for 15 min. Following permeabilization, cultures were washed with PBS and blocked with L15 media (GIBCO-BRL) plus 10% FCS for 30 min at room temperature. The coverslips were incubated with primary antibodies diluted in the blocking solution for 1 h at room temperature, washed in blocking solution and incubated for 1 h with species-specific affinity–purified rhodamine conjugated donkey anti–rabbit IgG or fluorescein-conjugated donkey anti–mouse IgG (Chemicon International, Inc., Temecula, CA) diluted in the blocking solution. Coverslips were washed in PBS, mounted in Citifluor (Citifluor Ltd., London, U.K.) and examined by epifluorescence with a Zeiss Axiophot microscope.

Immunoelectron Microscopy and Quantitation of Gold Particles

Myelinating cultures on collagen-coated aclar coverslips were rinsed in dPBS and fixed with 3.75% acrolein and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 min at room temperature. After fixation, the cultures were washed two times in 0.1 M phosphate buffer, pH 7.4, dislodged from the coverslips with forceps, and transferred to microfuge tubes containing the same buffer. The samples were spun for 15 min in a microfuge to pellet the cultures. The pellets were washed with phosphate buffer and embedded in LR White (The London Resin Co., Ltd., London, England). To prepare the samples for embedding, they were dehydrated by two 10-min incubations each of 50, 60, 70, and 80% ethanol. After the last dehydration step the pellets were incubated in two 1-h changes of a 1:1 mixture of 80% ethanol and LR White, four 30-min incubations with pure LR White, and an overnight incubation at 4°C in pure LR White. The following day the pellets were placed in tightly capped gelatin capsules filled with fresh LR White and incubated in a 50°C oven for 2 d to polymerize the resin.

Ultrathin sections (50–60 nm) were placed on 400-mesh nickel grids. For postembedding labeling the grids were subjected to five 2-min washes of PBSg (PBS containing 0.01 M glycine) and blocked for 10 min in PBSg containing 1% gelatin. Following five 1-min washes in PBSg the grids were floated, section facing down, on 20 μl drops of primary antibodies diluted in PBSg/0.5% BSA in a moist chamber at 37°C for 3 h. After eight 2-min washes in PBSg, the grids were incubated on 20 μl drops of 10 nm gold-conjugated goat anti–rabbit IgG (Amersham Intl., Arlington Heights, IL) diluted in PBSg/0.5% BSA for 90 min at room temperature. Unbound secondary antibody was removed by eight 2-min washes in PBSg and four 1-min washes in PBS. The grids were postfixed in 2% glutaraldehyde/PBS for 15 min, washed in PBS, and then incubated in 2% osmium tetroxide/PBS for 30 min. After washing in water, the grids were counterstained with uranyl acetate and lead citrate, washed, air dried, and analyzed on a Philips 201 electron microscope.

To assess the specificity of the anti-β4 immunolabeling, some grids were stained with antibody that had been preabsorbed with the immunogenic peptide. In these experiments, the peptide was preincubated with diluted affinity-purified anti-β4 antibody (16 μg/ml) for 2 h at room temperature at a final concentration of 400 μg/ml. This material was spun in a microfuge for 5 min prior to use.

The distribution of gold particles in the outer Schwann cell membrane, central region of the compact myelin, and periaxonal membrane was determined from micrographs of stained ultrathin sections. Tracings were prepared from 14 to 19 micrographs, depending on the region scored, and membrane lengths were then measured from these tracings with the aid of a computer drawing tablet and the Image 1.50 program (National Institutes of Health, Bethesda, MD). Gold particles within 0.04 μm of either side of the measured lines were scored. The mean value of gold particle densities

determined for each membrane was calculated from the measurements of individual micrographs and was analyzed by the unpaired *t* test.

Biotinylation of Surface Proteins and Immunoprecipitation

Surface proteins of primary Schwann cells and of myelinating cultures in 35 mm dishes were biotinylated as previously described (Rosen et al., 1992). Biotinylated cultures were extracted with 1% Triton X-100 in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF and 10 μg/ml each of antipain, pepstatin A, and leupeptin at 4°C (0.5 ml/dish). Lysates were spun in a microfuge for 20 min at 4°C and the cleared supernatants were preadsorbed with protein A Sepharose 4B (Sigma Immunochemicals) for 1 h at 4°C on a rotator. Proteins were immunoprecipitated by incubating 250 μl of preadsorbed lysate with 10 μl of each antiserum and 50 μl of packed protein A Sepharose for 1 h at 4°C on a rotator. The Sepharose was pelleted by spinning in microfuge and washed three times in lysis buffer containing protease inhibitors. Samples were boiled in sample buffer without β-mercaptoethanol and electrophoresed under nonreducing conditions on a 7.5% acrylamide SDS gel. The gel was blotted onto nitrocellulose (Towbin et al., 1979) and probed with alkaline phosphatase–conjugated streptavidin (Bio-Rad Laboratories, Richmond, CA). Biotinylated proteins were detected by developing the blot with 5-bromo-4-chloro-3-indoyl-phosphate and Nitroblue tetrazolium (Kirkegard and Perry Laboratories, Inc., Gaithersburg, MD).

Cultured Cell and Tissue Lysate Preparation

Total SDS lysates of sciatic nerves were prepared by two different methods. In the first method sciatic nerves were removed from Sprague Dawley rat pups of different ages or from adult rats, frozen immediately on dry ice, and then stored at −80°C. To prepare lysates the nerves were homogenized by sonication in lysis buffer (95 mM NaCl, 25 mM Tris-Cl, pH 7.4, 10 mM EDTA, 2% SDS, 1 mM PMSF, and 10 μg/ml each of antipain, pepstatin A, and leupeptin) and then incubated in a boiling water bath for 5 min. In the second method, the nerves were immersed in liquid nitrogen immediately after dissection and ground into fine pieces. The liquid nitrogen was then removed, the lysis buffer was added and the samples were heated as above. Insoluble material from samples prepared by both methods was pelleted by centrifugation of the samples in a microfuge for 10 min at room temperature. Lysates of Schwann cell cultures grown in the presence or absence of GGF and forskolin, and myelinating Schwann cell/neuron cocultures, were prepared by scraping the cells in SDS lysis buffer. The lysates were heated and spun in a microfuge to remove insoluble material as described above. The protein concentrations of the cleared supernatants were determined by the Micro BCA method (Pierce, Rockford, IL). Lysates were subjected to SDS gel electrophoresis and blotted onto nitrocellulose. Blots were probed with antibodies against β4 and β1 followed by ^{125}I-labeled protein A (Amersham Intl.) and exposed for autoradiography. Quantitation of the immunoreactive bands on the blots was performed on a Molecular Dynamics phosphorimager. Autoradiographs were scanned with a Truvell scanner at 600 dots per inch (dpi), and images were cropped with Adobe Photoshop. QuarkExpress was used for page layout to create images that were printed by a commercial typesetter on a linotronics printer at 2,540 dpi and a 150 line per inch halftone screen.

Results

Axonal Regulation of Schwann Cell Integrin Expression

The pattern of expression of two integrin subunits, β1 and β4, by Schwann cells alone and by myelinating Schwann cells in coculture with neurons, was characterized. We first determined the expression of the β1 and β4 subunits by primary Schwann cells using immunofluorescent techniques. As shown in Fig. 1, in the absence of neurons, primary Schwann cells express significant levels of the β1 integrin but barely detectable levels of β4. Immunofluorescent staining of sensory neurons treated to eliminate nonneuronal cells (Fig. 2) similarly demonstrate that these neurons do not express β4
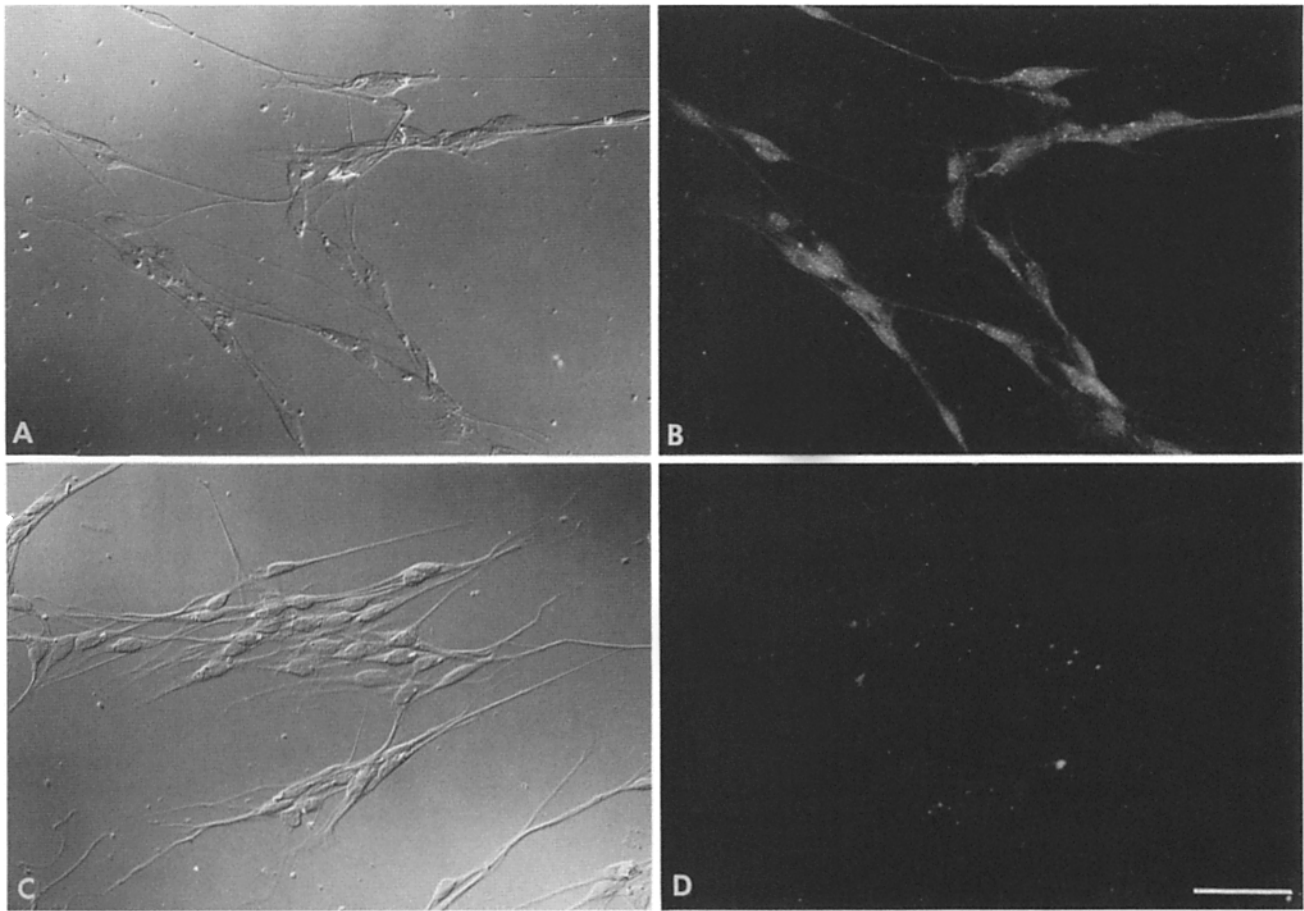
Figure 1. Expression of β1 and β4 integrins by primary Schwann cells. Primary rat Schwann cells were grown on collagen-coated coverslips and immunostained, after fixation and permeabilization, with antibodies specific for the carboxy terminus of β1 (*B*) or β4 (*D*). Nomarski images of the corresponding fields are shown in *A* and *C*. Bar, 50 μm.

(there is faint staining of the neuronal somas due to nonspecific staining by the secondary antibody), although they do express modest levels of β1 by immunostaining (data not shown).

We next examined the expression of β4 by Schwann cells in coculture with neurons. Ascorbic acid was added to promote ensheathment and myelination. At various times thereafter the cultures were fixed and double stained for β4 and MAG, a myelin specific protein which is expressed at the onset of myelination (Owens and Bunge, 1989). Three days after adding ascorbic acid, a few myelin segments, which are just beginning to form, are present in the cultures (Fig. 3, *A–C*). These nascent sheaths are typically shorter than the more mature internodes found in older cultures. They appear to express MAG throughout their thickness, possibly due to the presence of MAG in the uncompacted membranes of the forming myelin sheath (Trapp, 1988). Interestingly, the same segments also express β4 at high levels with a distribution that precisely overlaps MAG. Other Schwann cells in the field, which are in contact with neurites but have not formed myelin, express β4 at much lower levels, if at all. These results indicate that expression of β4 markedly increases at the onset of myelination. They also suggest that MAG and

β4 may initially be present together in the uncompacted membranes of the nascent myelin sheath.

At later times, i.e., at 8 and 14 d after adding ascorbic acid (Fig. 3, *D–F* and *G–I*, respectively), MAG and β4 acquire distinct distributions. The myelin sheaths at this stage are considerably longer as visualized by Nomarski or immunofluorescent staining. MAG and β4 are present in the same myelinated segments but MAG is typically expressed along the entire length of the myelin internode as a thin line of staining. This staining pattern is consistent with its expression at high levels in the periaxonal membrane (Trapp and Quarles, 1982; Martini and Schachner, 1986). By contrast, β4 appears to stain the outside of the entire myelin sheath suggesting that it is present in the outer plasma membrane of the myelinating Schwann cell (see also below).

Interestingly, β4 also accumulates in Schmidt-Lanterman clefts, a site where MAG is known to be concentrated (Trapp and Quarles, 1982). These structures become progressively more prominent as the myelin sheaths mature (Fig. 3, *H* and *I*, *arrows*). The accumulation of MAG in the Schmidt-Lanterman clefts occurs earlier and is more consistent than that observed for β4 (compare the intensity of staining of MAG and β4 in the incisures, Fig. 3, *E* and *F*, *arrows*). MAG

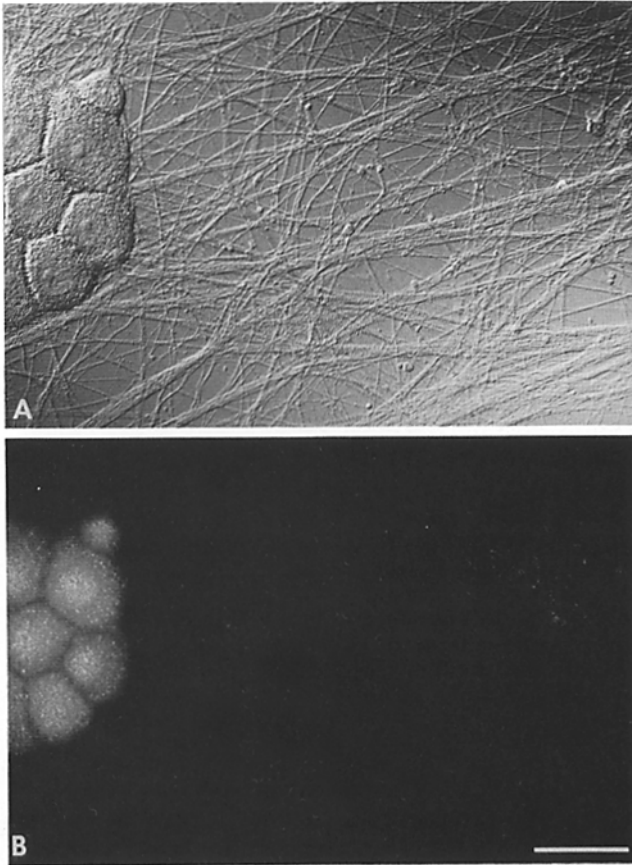*Figure 2.* Absence of β4 expression by primary sensory neurons. Dissociated DRG neurons were fixed and stained with anti-β4 antibodies (*B*); the Nomarski image of the same field is shown in *A*. Note the absence of neurite staining. The staining of the neuronal somas is nonspecific and was also observed in control samples stained with the fluorescent secondary antibody alone (data not shown). Bar, 50 μm.

and β4 remain concentrated in Schmidt-Lanterman clefts more than one month after adding ascorbic acid (data not shown), suggesting that both proteins persist in these sites. MAG also becomes concentrated in the paranodal loops which are located on either side of the nodes of Ranvier (Fig. 3, *H* and *I, open arrow*) as previously described (Trapp and Quarles, 1982), in contrast to β4 which does not appear to accumulate to the same extent in these structures.

These immunofluorescent studies suggest that β4 expression in Schwann cells requires axonal contact. To seek additional evidence for the axonal regulation of β4 expression, we performed immunoblot analysis. As shown in Fig. 4 *A* (lane *a*), Schwann cells alone express negligible levels of β4. When Schwann cells are cocultured with neurons under conditions promoting myelination, they express high levels of β4 (Fig. 4 *A*, lane *c*). In addition to the 200-kD β4 band, a smaller band of approximately 180 kD was recognized by the antibody which probably corresponds to a proteolytic fragment of β4 (discussed further below). We also treated Schwann cells with forskolin and GGF to elevate their cAMP levels and analyzed their expression of β4. (Many of the effects of the axon on Schwann cells, including the induction of myelin specific proteins, can be mimicked by treating

confluent cultures of Schwann cells with agents that elevate intracellular levels of cAMP [Jessen and Mirsky, 1991].) Under these conditions, we also detected a small amount of the degraded form of β4 (Fig. 4 *A, arrowhead*).

To determine whether the expression of β4 requires continued axonal contact, we placed undissociated DRG, treated to eliminate fibroblasts, into culture and allowed them to myelinate for one month. By using explant cultures, all of the cell somas can be removed by mechanical dissection of the explant, thereby initiating Wallerian degeneration in vitro. After removing neuronal somas from such cultures, neurites rapidly degenerate and Schwann cells, which had previously formed myelin, specifically and reliably proliferate during the ensuing few days (Salzer and Bunge, 1980). To identify Schwann cells which had previously formed myelin and expressed β4 at high levels, we incubated such cultures with BrDU for several days after axotomy to label proliferating cells. We found that Schwann cells, which incorporated BrDU into their nuclei and were breaking down their myelin, exhibited significantly lower levels of β4 four days after axotomy (Fig. 5, *C* and *D*). By contrast, myelinating Schwann cells in control explants from the same dish continued to express β4 at high levels and had not incorporated BrDU into their nuclei (Fig. 5, *A* and *B*). These studies indicate that, like myelin proteins, expression of β4 requires continued contact with the axon. In addition, as the basal lamina typically persists for a week or more after axotomy in vitro (Bunge et al., 1982), these findings suggest that Schwann cell contact with components of the basal lamina is not sufficient by itself, in the absence of axonal contact, to maintain β4 expression.

In contrast to β4 expression, β1 expression by Schwann cells does not increase as a consequence of axonal contact or myelination. Immunofluorescent staining demonstrates that β1 is constitutively expressed by primary Schwann cells (Fig. 1). Expression of β1 does not appear to increase, and may even decrease, when Schwann cells are cocultured with neurons. This is evident in Fig. 6 in which the expression of MAG and β1 are compared. β1 appears to be expressed at the surface of all Schwann cells, including those that have formed myelin, but in contrast to β4, there is not a striking difference in the level of β1 expression between myelinated and ensheathing Schwann cells. Immunoblot analysis also demonstrates that primary Schwann cells express high levels of β1 (Fig. 4 *B*, lane *a*). Of note, the level of β1 expression in Schwann cells treated with GGF and forskolin (Fig. 4 *B*, lane *b*) is approximately 40% less than in untreated Schwann cells. Taken together, these results suggest that β1 and β4 expression are inversely regulated during Schwann cell myelination.

Identification of Associated Alpha Subunits

While β4 is believed to combine exclusively with α6, β1 can associate with a wide variety of α subunits (Hynes, 1992). We therefore used immunoprecipitation to identify the major subunit combinations expressed by Schwann cells and to study their modulation during myelination in vitro. Cell surface proteins from cultures of Schwann cells alone (Fig. 7 *A*) or from myelinating Schwann cells in coculture with neurons (Fig. 7 *B*) were biotinylated, extracted with detergent, and immunoprecipitated with β1-, β4-, or α6-specific antibodies
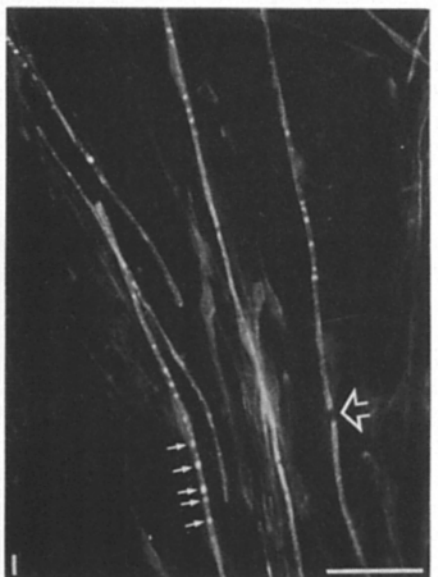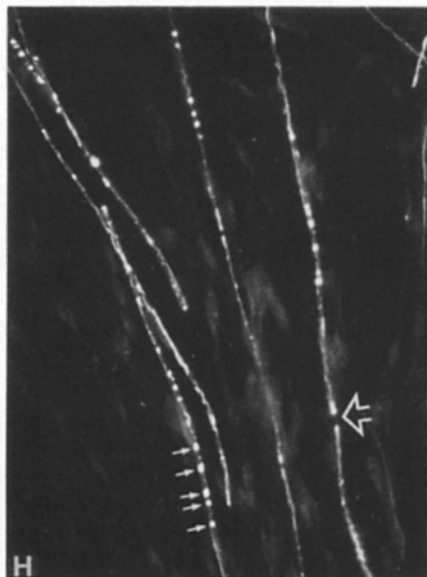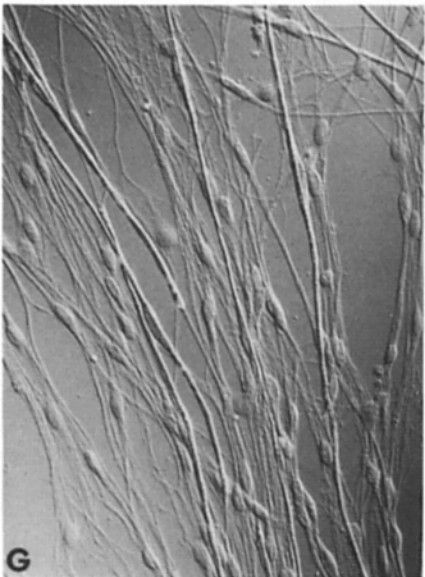
A   B

a b c   a b c

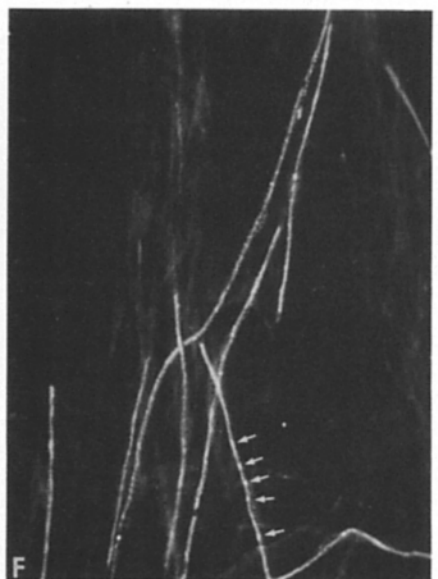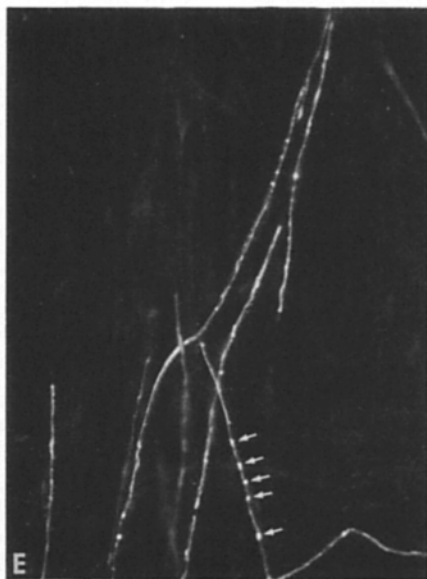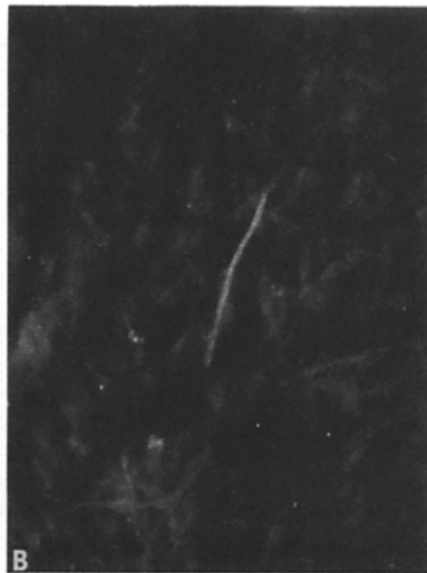*Figure 4.* Immunoblot analysis of β1 and β4 expression in Schwann cells and Schwann cell/neuron cocultures. 75 μg of protein lysate prepared from untreated Schwann cells (lanes *a*), Schwann cells treated with GGF and forskolin for 2 wk (lanes *b*), or myelinating Schwann cell/neuron cocultures (lanes *c*) were fractionated by SDS PAGE, blotted onto nitrocellulose, and probed with an antibody specific for β4 (Fig. 4 *A*) or for β1 (Fig. 4 *B*). Blots were incubated with ^{125}I protein A and exposed for autoradiography for 6 d (Fig. 4 *A*) or for 15 h (Fig. 4 *B*). The arrow in *A* indicates the position of the 180-kD fragment of β4.
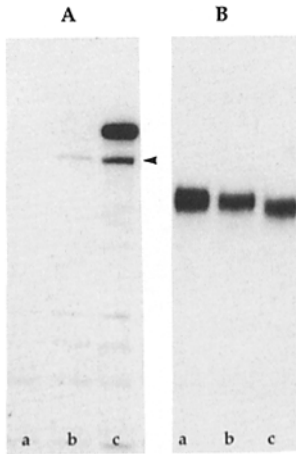
(Fig. 7, lanes *a–c*, respectively). In cultures of Schwann cells alone, we found that β1 is associated predominantly with α6 (compare the intensity of β1 in lanes *a* and *c* of Fig. 7 *A*), and to a lesser extent with α1. The identity of α1 was inferred by its size and confirmed by immunoprecipitation with anti-α1 specific antibodies (data not shown). Other alpha subunits that are known to be associated with β1 in Schwann cells (Toyota et al., 1990; Hsiao et al., 1991; Lefcort et al., 1992; Jaakkola et al., 1993), were not specifically investigated in these studies but would appear to be present at reduced levels. Essentially no β4 is detectable in primary Schwann cells, consistent with the results shown in Figs. 1 and 4. Also consistent with this result, immunoprecipitation of α6 (and α1) from cultures of primary Schwann cells, coprecipitates β1 but not β4.

By contrast, in myelinating cultures β4 was abundantly expressed and apparently exclusively associated with α6 (Fig. 7 *B*, lane *b*). Immunoprecipitation of α6 (Fig. 7 *B*, lane *c*) demonstrates that it is associated with β4, as well as some β1. The α6β4 complex is likely to be present in myelinating Schwann cells which, as shown above, express significantly higher levels of β4 than β1. In contrast, α6β1 is likely to be present primarily in the ensheathing, nonmyelinating Schwann cells, which express β1 at high levels relative to β4. We also cannot rule out a contribution from the sensory neurites, which express both α1β1 and α1β6 (data not shown), although they are likely to be less accessible to a surface label under the conditions of this study. In addition, we have detected the 180-kD fragment of β4 in these cultures (Fig. 7, lane *b*), which we also observed in the immunoblots (see Fig. 4). This β4 fragment was biotinylated and immunopre-

cipitated with an antibody specific for the extreme carboxy terminus of β4, indicating that it is expressed at the cell surface and must lack a portion of the amino terminus, respectively. It is of note that this β4 fragment no longer associates with α6 as evidenced by its inability to be coprecipitated by α6 antibodies. Finally, these findings also suggest that the biosynthesis of α6 is not significantly modified during myelination. However, while α6 is associated with β1 in primary Schwann cells, in myelinating cells it is associated with the newly synthesized β4.

It should be noted that the α6 antibody used in these experiments specifically recognizes the alternatively spliced A variant. α6A appears to be the predominant α subunit associated with β1 and β4 as evidenced by the comparable intensity of α6 in Fig. 7 *A*, lanes *a* and *c* and in Fig. 7 *B*, lanes *b* and *c*. These findings are consistent with the recent study of Hogervorst et al. (1993) in which α6A, but not α6B, was detected in Schwann cells of peripheral nerve.

Differential Expression of β1 and β4 during Peripheral Nerve Development

Consistent with the different patterns of β1 and β4 expression by primary and myelinating Schwann cells, we have found that expression of these two integrin subunits also differs during peripheral nerve development in vivo. Sciatic nerves were removed from animals of various postnatal ages and immediately frozen on dry ice prior to sonicating and boiling in 2% SDS. Alternatively, the tissues were directly frozen and fragmented in liquid nitrogen prior to boiling in the SDS buffer. Equal amounts of protein from each time point were loaded per lane, and representative immunoblots are shown in Fig. 8.

These studies demonstrate that β4 immunoreactivity increases approximately threefold during peripheral nerve development, reaching a maximum by day 25 and then declining slightly (Fig. 8, *upper panel*). This pattern roughly parallels the time course of myelination in the peripheral nervous system (Wood and Engel, 1976), although the absolute increase of β4 expression is significantly less and more delayed than that observed for myelin proteins, such as MAG (Pedraza et al., 1991). In striking contrast to the increase in β4 expression, β1 levels in peripheral nerve progressively and dramatically decrease with time (Fig. 8, *lower panel*). Although these studies are complicated by the contribution of other peripheral nerve components, notably from the axon and the perineurial cell, these results are consistent with the culture studies described above which demonstrated an increase in β4 but not in β1 levels with myelination.

A large fraction of the β4 subunit present in peripheral nerve may be proteolyzed. This is most evident at later times of development when, in addition to a band of 200 kD (cor-

Figure 3. MAG and β4 expression in Schwann cells are induced by axonal contact. Schwann cells were added to cultures of dissociated sensory neurons and allowed to repopulate the neurites. Ascorbic acid was added to promote myelination and cultures were then fixed after an additional 3 d (*A–C*), 8 d (*D–F*), or 14 d (*G–I*). Cultures were immunostained for MAG (*B*, *E*, and *H*) and β4 (*C*, *F*, and *I*). Corresponding Nomarski images are shown in *A*, *D*, and *G*. At 3 d, MAG and β4 are prominently expressed in a few segments of myelin that are beginning to form. 8 d after adding ascorbate, MAG is readily visible in presumptive Schmidt-Lanterman clefts (*E*, arrows), in contrast to β4 which is not as concentrated. By 14 d, both MAG and β4 accumulate into the same clefts (*H* and *I*, arrows). MAG staining is thinner than β4 in most myelin segments and is more concentrated on either side of the nodes of Ranvier (*H* and *I*, open arrows) than is β4. Bar, 50 μm.
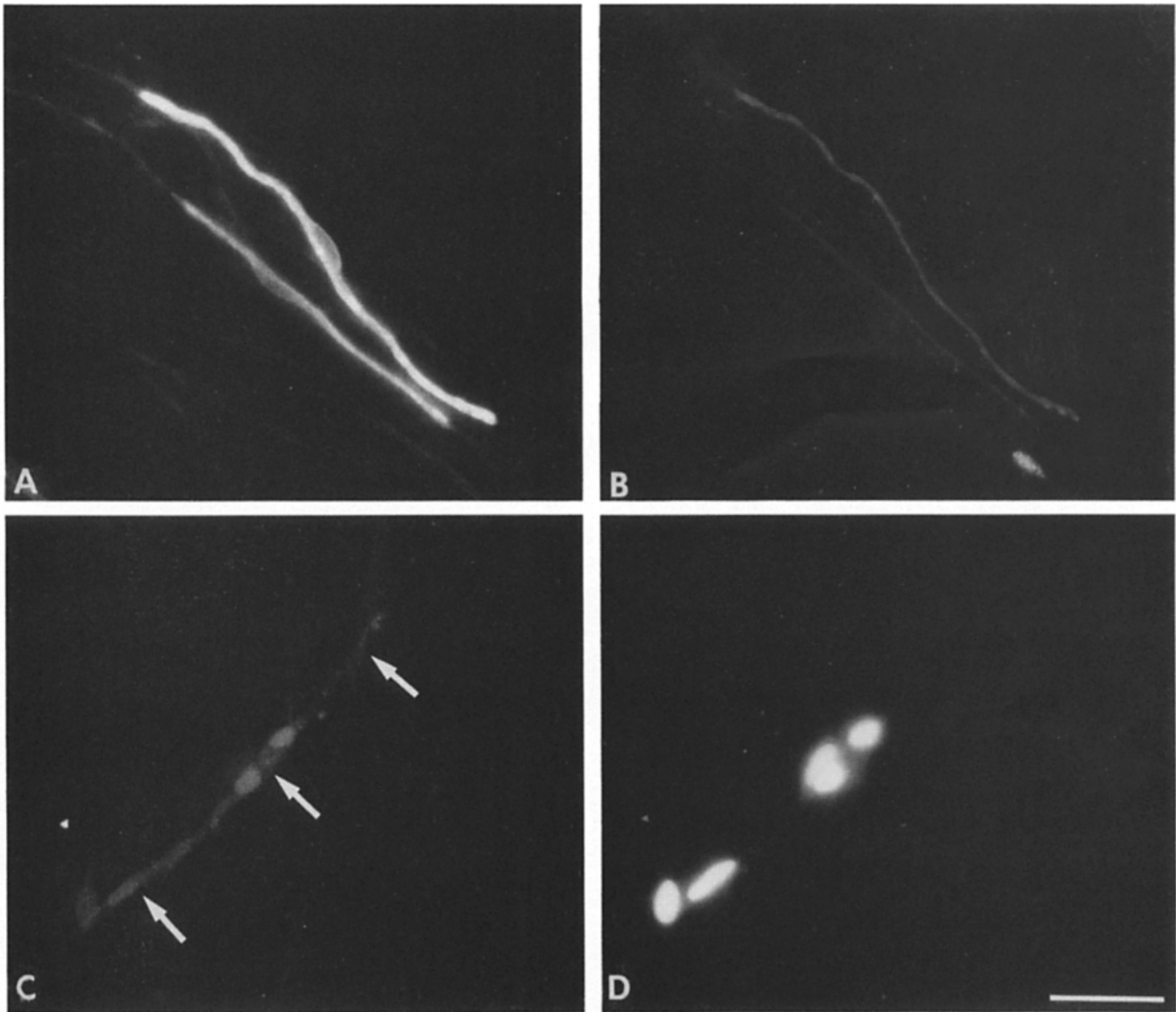
Figure 5. Axonal dependence of β4 expression. Myelinated fibers from a control DRG explant (*A* and *B*) or a myelinated fiber undergoing Wallerian degeneration 4 d after excision of its ganglion (*C* and *D*) are shown; each field is from a different area of the same coverslip. Immunofluorescent staining demonstrates that the two myelin sheaths in the control explant express β4 at high levels (*A*) whereas the myelinated fiber undergoing Wallerian degeneration, indicated by the arrows in *C*, only faintly stains for β4. Cultures were incubated with BrDU for 3 d to label the nuclei of previously myelinating Schwann cells which brightly stain in *D*, and are unstained in the control field (*B*). Bar, 50 μm.

responding to the intact protein), bands of 180 and 80 kD are also detected despite efforts to rapidly inactivate proteases immediately after sciatic nerve dissection. Preabsorption of the affinity-purified antibody with a synthetic peptide corresponding to the carboxy terminus of β4 abolished reactivity with these bands suggesting that they are indeed proteolytic fragments of β4 (data not shown). Of note, the relative abundance of β4 and its fragments appears to be developmentally regulated. Most of the β4 present at birth appears to be intact whereas at later developmental stages the proteolytic fragments are most abundant. This developmental profile was highly reproducible and present whether nerves were initially frozen on dry ice or frozen directly in liquid nitrogen prior to detergent extraction. (In contrast to β4, the β1 present in peripheral nerve is intact at all postnatal

stages, being comprised of either full length β1 or preβ1, suggesting that the β4 fragments present are specific and do not result from nonspecific proteolysis accompanying sample preparation.) These results are consistent with previous studies demonstrating that β4 is partially proteolyzed in both its extracellular and intracellular domains, in the latter case apparently via the action of an endogenous calpain (Giancotti et al., 1992).

Ultrastructural Localization of β4 to the Outer Plasma Membrane of Schwann Cells

The immunofluorescent studies described earlier suggest that β4, in mature myelin sheaths, is concentrated in the outer plasma membrane of the Schwann cell and in some
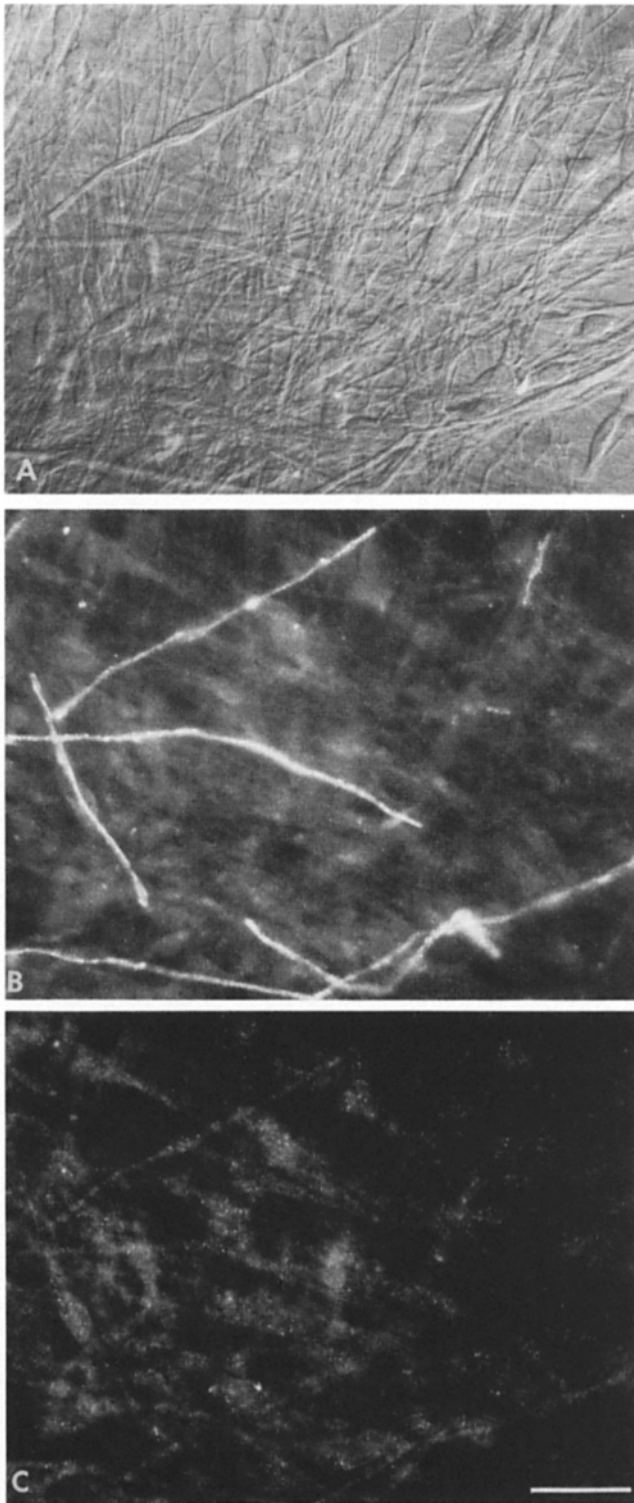
Figure 6. β1 expression in myelinated Schwann cell cultures. Myelinating cultures were visualized by Normarski microscopy (*A*), or by immunofluorescence following staining with a monoclonal antibody to MAG (*B*), or a polyclonal antibody to β1 (*C*). Myelinated fibers, which express MAG at high levels, are faintly stained with the anti-β1 antibody. The amount of β1 staining of the myelinated segments appears to be comparable to the staining present on non-myelinating Schwann cells present throughout the microscopic field. Bar, 50 μm.
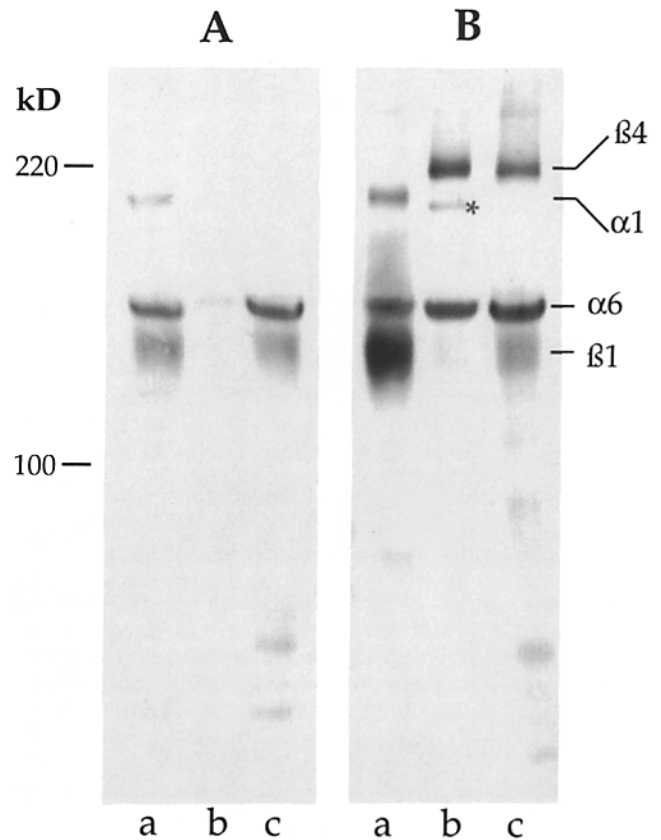

Figure 7. Identification of the α subunits associated with β4 and β1 in Schwann cells and myelinating Schwann cell/neuron cocultures. Detergent extracts of surface biotinylated cultures of Schwann cells (*A*) or of myelinating Schwann cell cultures (*B*) were immunoprecipitated with antibodies against β1 (lane *a*), β4 (lane *b*), and α6 (lane *c*). The position of the corresponding precipitated integrin subunits are indicated to the right of *B*. The asterisk in lane *b* of *B* marks a putative proteolytic fragment of β4 that was not precipitated with antibodies against α6 (*B*, lane *c*).

Schmidt–Lanterman clefts. The localization of β4 in the outer plasma membrane is suggested by the outlining of the myelin sheath seen in Fig. 3 and is consistent with the abundant biotinylation of β4 under conditions expected to primarily label cell surface proteins.

To confirm that β4 is expressed at the surface of the Schwann cell in close association with the basal lamina, we have performed immunoelectron microscopy, and compared the distribution of β4 to that of MAG and MBP, which are known to be localized to the periaxonal membrane and compact myelin, respectively (Martini and Schachner, 1986; Trapp et al., 1989). Results are shown in Fig. 9. These studies demonstrate that most, if not all of the β4 is present in the outer plasma membrane in contact with the basal lamina (Fig. 9, *A* and *B*). This pattern of β4 staining was observed both in acrolein fixed material and in glutaraldehyde fixed material and was blocked by preabsorption of the antibody with a synthetic peptide to the carboxy terminus of β4 (see below). By contrast, MAG and MBP are concentrated in the periaxonal membrane and compact myelin, respectively (Fig. 9, *C* and *D*), demonstrating that these membrane compartments were fully accessible to the β4 antibody and
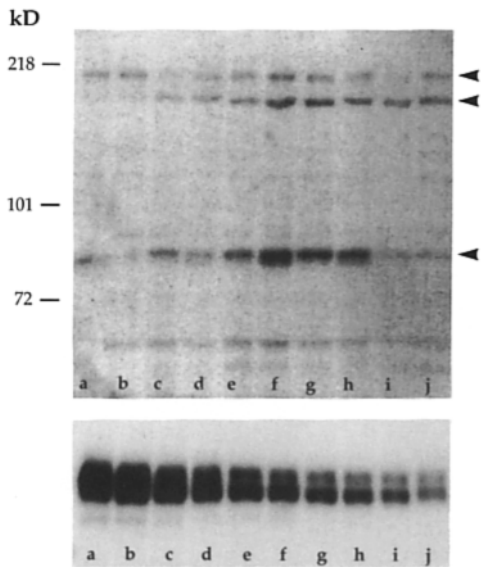
Figure 8. Expression of β1 and β4 integrins during peripheral nerve development. 75 μg of protein lysate, prepared from sciatic nerves of rats of different ages, were fractionated by SDS PAGE and blotted onto nitrocellulose. Lanes *a–j* correspond to sciatic nerves isolated at postnatal days 1, 3, 7, 10, 15, 20, 24, 30, 41, and the adult respectively. (*Upper panel*) Expression of β4 during peripheral nerve development. Arrowheads indicate bands that specifically react with the β4 antibody. Reactivity with these bands was lost after preabsorption of the β4 antibody. (*Lower panel*) Expression of β1 during peripheral nerve development.

confirming the appropriate distribution of these myelin-specific proteins in this culture system.

Quantitation of the immunogold labeling of β4 in different membranes of the myelinating Schwann cell is presented in Table I. These results demonstrate that specific staining of β4 is only detected in the outer plasma membrane, and this staining is abolished by preabsorption of the antibody with the β4 peptide.

Discussion

Differential Expression of Schwann Cell Integrins

In this paper, we have investigated the expression of integrins by Schwann cells to clarify the mechanisms by which the basal lamina promotes the ensheathment and myelination of axons. We have shown that the expression of integrins by Schwann cells is under axonal control. Whereas primary Schwann cells constitutively express high levels of β1 but do not express detectable levels of β4, myelinating Schwann cells express significant levels of β4 and appear to reduce their expression of β1. Thus α6β1 and α6β4 are abundantly expressed by primary Schwann cells and by myelinating Schwann cells, respectively. In all conditions examined, Schwann cells may also express low levels of α1β1 as previously reported (Toyota et al., 1990). Other α subunits which associate with β1 and are known to be expressed by Schwann cells in peripheral nerve (namely α2, α3, and α5) (Toyota et al., 1990; Hsiao et al., 1991; Lefcort et al., 1992; Jaakkola et al., 1993) were not characterized in these studies.

These studies indicate that during Schwann cell differenti-

ation, there is a transition in expression from β1 integrins, particularly α1 and α6, which predominate initially, to the α6β4 integrin, which predominates in myelinated Schwann cells. This is also suggested by the differential effects of GGF and forskolin on the expression of these integrins by Schwann cells and is consistent with the striking difference in the developmental profiles of these β subunits during sciatic nerve development as shown in Fig. 8. These results differ somewhat from a recent immunofluorescent study of the expression of β1 and β4 integrin during development of human sciatic nerve in which these integrins were found to be transiently expressed by embryonic Schwann cells, and persistently expressed by embryonic perineurial cells (Jaakkola et al., 1993). However, they are consistent with other studies showing persistent expression of β4 by Schwann cells in adult human (Hogervorst et al., 1993), murine (Sonnenberg et al., 1990), and rat (S. Einheber and J. Salzer, unpublished observations) peripheral nerves by immunocytochemistry.

These results are also in agreement with recent in vivo studies in which the expression of β4 mRNA in peripheral nerve was reported to be axon dependent (Feltri et al., 1992). Taken together these studies indicate that, as in the case of the axonal regulation of myelin protein expression (Lemke and Chao, 1988), the increase in β4 expression during myelination is transcriptionally regulated. We have also found that expression of the β4 subunit is rapidly downregulated after removing neurons from these cultures despite the expected persistence of the basal lamina. A similar decrease in Schwann cell β4 expression has been observed during Wallerian degeneration in vivo (L. Feltri, S. Scherer, and L. Wrabetz, personal communication). These results suggest that continuous expression of β4 also requires axonal contact.

Potential Significance of the Differential Expression of β1 and β4

β1 and β4 integrins differ in several important ways. α6β1 and α1β1 are prototypic and well-characterized integrins that are expressed by a wide variety of cell types (see Hynes, 1992 for a brief review). α6β1 binds to laminin and α1β1 is a dual collagen/laminin receptor (Kramer and Marks, 1989; Hall et al., 1990). Like other integrins, they are believed to mediate the interaction of the actin cytoskeleton to the inner face of the membrane at points of cell contact with their extracellular ligands. By contrast, α6β4 is an unusual integrin in several regards (Quaranta and Jones, 1991). It has a more restricted distribution being principally expressed by epithelial cells, Schwann cells, perineurial cells, and a limited subset of endothelial cells (Sonnenberg et al., 1990; Kennel et al., 1992). β4 is only known to associate with the α6 subunit (Hemler et al., 1989). Unlike other β subunits, β4 contains an unusually long cytoplasmic domain of more than 1,100 amino acids that is alternatively spliced and contains two pairs of fibronectin type III repeats (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990).

The role of α6β4 has been best characterized in the hemidesmosome of epithelial cells where it is a major component (Stepp et al., 1990; Sonnenberg et al., 1991). Hemidesmosomes also contain the bullous pemphigoid antigens, the keratin intermediate filaments (Legan et al., 1992), and were recently found to contain several high molecular weight pro-
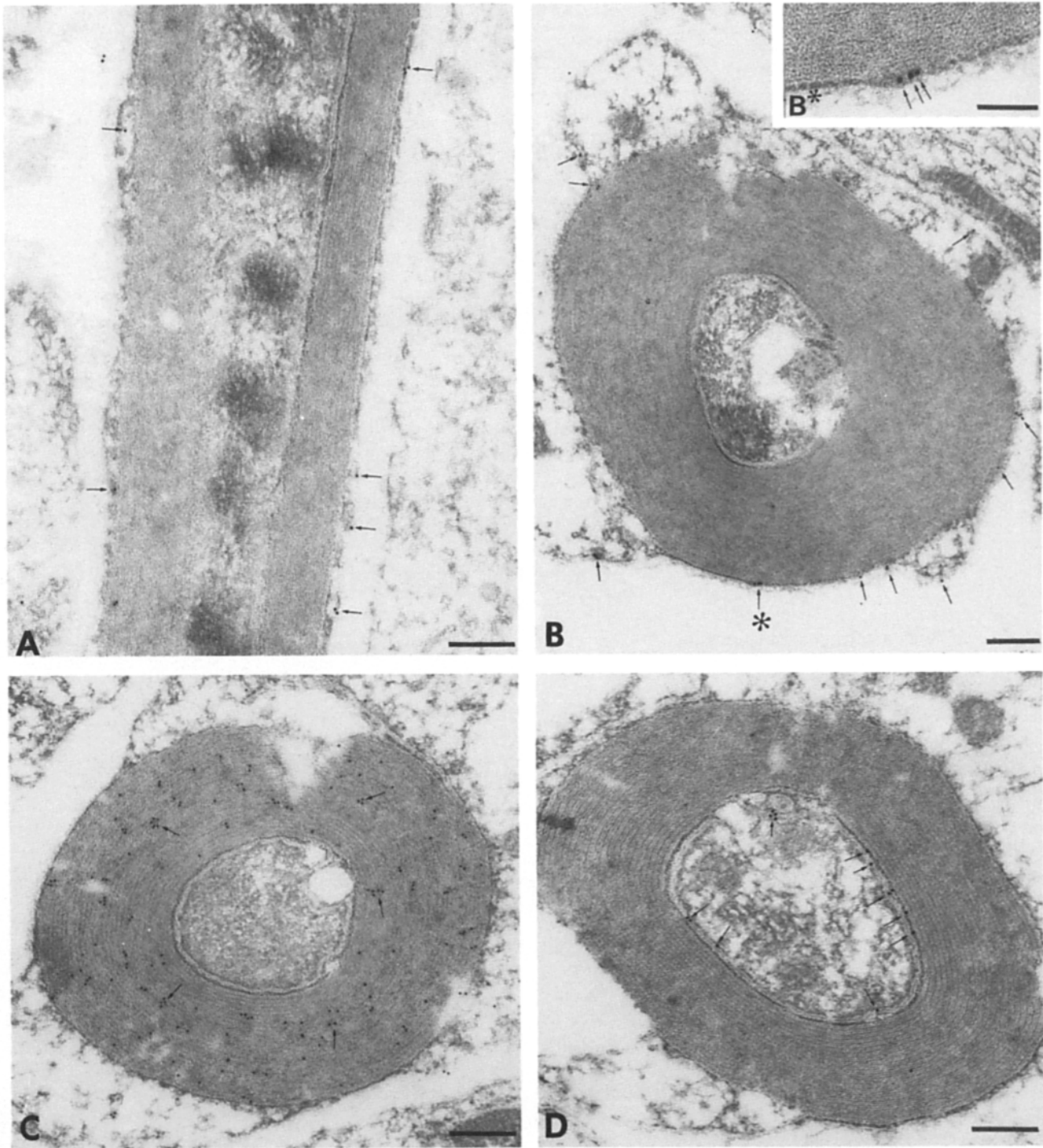
Figure 9. Immunoelectron microscopic localization of β4, MBP, and MAG in myelinating Schwann cell cultures. Cultures were fixed 21 d after adding ascorbic acid to initiate myelination. The location of the β4 integrin subunit was identified in a longitudinal section (*A*) or cross section (*B*) of a myelinated axon by immunolabeling. Gold particles associated with the outer plasma membrane are indicated by the arrows. One such area, indicated by the asterisk in *B*, is shown at higher magnification in the inset. Cross sections of myelinated fibers from the same cultures that were immunolabeled for MBP (*C*) and MAG (*D*) are shown. MBP is present throughout the compact myelin (a few representative gold particles are demarcated by the *arrows*) whereas MAG is concentrated in the periaxonal membrane as indicated. Bars: (*A–D*) 0.25 μm; (inset) 0.1 μm.

teins including one termed HD1 (Hieda et al., 1992). Expression of β4 appears to be critical for the assembly of this complex as antibodies to β4 block the formation of hemidesmosomes in vitro (Jones et al., 1991). The molecules with which α6β4 directly interacts in the hemidesmosome have not yet been identified. α6β4 is thought to bind to one of the extracellular matrix proteins of the basal lamina, possibly laminin (Lee et al., 1992), although this is controversial (Sonnenberg et al., 1990); its actual ligand in situ is not yet known. α6β4 is also believed to interact, either directly

Table I. Analysis of β4 Immunogold Labeling

Antibody	Schwann cell membrane	Membrane length (μm)	Number of particles	Mean particles/μm
Anti-β4	Outer pm	204.5	205	1.01 ± 0.07
	Myelin	115.8	8	0.08 ± 0.04
	Periaxonal	54.3	3	0.08 ± 0.05
Preabs anti-β4	Outer pm	186.5	14	0.07 ± 0.03
	Myelin	101.4	1	0.01 ± 0.01
	Periaxonal	52.8	3	0.07 ± 0.05

The density of gold particles (mean particles/μm) in the outer plasma membrane (outer pm), compact myelin, and periaxonal membrane were determined from electron micrographs of myelinating Schwann cells immunolabeled with anti-β4 antibody or preabsorbed antibody. The membrane length and the number of gold particles scored represent total values measured from a set of micrographs. The mean number of gold particles/μm of membrane ±SEM was calculated from the particle densities determined for each micrograph. The density of gold particles in the outer plasma membrane labeled with anti-β4 was significantly greater than that in compact myelin and periaxonal membrane labeled with anti-β4 or the outer plasma membrane labeled by preabsorbed antibody ($P < 0.0001$).

or indirectly, with the keratin filaments via its unusually long cytoplasmic domain (Quaranta and Jones, 1991). α6β4 therefore differs from other integrins, notably α6β1, by interacting with intermediate filaments rather than with the actin cytoskeleton.

We have not observed hemidesmosome-like structures associated with the α6β4 staining in these cultures, although subplasmalemmal densities in regions of the Schwann cell apposed to the basal lamina have previously been reported (Bunge et al., 1982). The absence of typical hemidesmosomes in peripheral nerve is also consistent with the apparent lack of bullous pemphigoid antigen (Hogervorst et al., 1993) or of keratin intermediate filaments in myelinating Schwann cells, although HD1 has been reported to be expressed by Schwann cells (Hogervorst et al., 1993). In addition, rather than being concentrated in hemidesmosomes, we have found that β4 is diffusely expressed at the surface of myelinating Schwann cells. It is therefore likely to be interacting with components in myelinating Schwann cells that differ from those present in the hemidesmosome of epithelial cells.

Although there are no keratin filaments in Schwann cells, α6β4 could potentially interact with other intermediate filaments present in Schwann cells. It is of interest in this regard that Schwann cells express different sets of intermediate filaments depending on their state of differentiation. Thus, nonmyelinating Schwann cells express glial fibrillary acidic protein (GFAP) (Jessen et al., 1990) and vimentin, whereas myelinating Schwann cells do not express GFAP, but instead appear to express neurofilament subunits (the middle and light chains) (Kelly et al., 1992; Roberson et al., 1992) and vimentin (Kelly et al., 1992). It is not yet known if α6β4 is associated with neurofilaments or other intermediate filaments of myelinating Schwann cells. However, it may be relevant that as the Schwann cell makes a transition from an ensheathing state to a myelinated state, a transition that involves a dramatic change in the shape of the Schwann cell that is likely to involve the remodeling of its cytoskeleton (Kelly et al., 1992), it also apparently switches its expression of integrins. Whether this transition from β1 to β4 reflects a change in the linkage of the extracellular matrix from an actin based cytoskeleton in ensheathing cells to an intermediate filament based one in myelinating cells, is an important question for future investigation.

Interactions of Schwann Cells with the Extracellular Matrix

The immunolocalization studies reported here indicate that α6β4 is present in the outer plasma membrane of myelinating Schwann cells in close association with the basal lamina (see Fig. 9 and Table I). This is also consistent with the biochemical studies which demonstrated that α6β4 is accessible to an impermeant surface label (see Fig. 7). Initially MAG and β4 appear to be present in the same membranes in the nascent myelin sheath, whereas in the mature sheath they have distinct distributions. The differential distributions of these proteins effectively create two distinct membrane domains of the Schwann cell: an adaxonal membrane in which MAG is concentrated and an abaxonal membrane in which β4 is concentrated. The distinct distribution of these proteins may be critical for the ability of the Schwann cell to establish an appropriate cell polarity which, in turn, may be required for assembly of the myelin sheath (Bunge et al., 1986).

The localization of β4 in the outer plasma membrane could reflect its binding to a component of the Schwann cell basal lamina. This basal lamina is known to contain collagen types IV and V, entactin, laminin, a heparan sulfate proteoglycan, and possibly fibronectin (see Bunge et al., 1986 for review). Axonal contact upregulates Schwann cell expression of type IV collagen (Carey et al., 1983), the heparan sulfate proteoglycan (Eldridge et al., 1986), and it is critical for the assembly of a basal lamina by Schwann cells (Clark and Bunge, 1989). In addition, while primary Schwann cells synthesize laminin constitutively (Cornbrooks et al., 1983; Baron-Van Evercooren et al., 1986), laminin is redistributed from a patchy expression pattern on the surface of Schwann cells in the absence of axonal contact, to a highly uniform expression pattern as a result of axonal contact (Cornbrooks et al., 1983).

Laminin has a particularly striking effect on Schwann cell behavior. Changes in Schwann cell morphology and basal proliferation rates have been observed when these cells are cultured on a laminin substrate (Baron-Van Evercooren et al., 1986). Of considerable interest, laminin has been shown to correct the Schwann cell ensheathment and myelination defect that occurs in defined media, whereas type IV collagen, for example, cannot (Eldridge et al., 1989). It may therefore be relevant that the two major integrins expressed during Schwann cell differentiation, i.e., α6β1 and α6β4, have both been proposed to be laminin receptors. Whether laminin is able to promote Schwann cell ensheathment via the α6β1 integrin, via α6β4, or via another, as yet unidentified, integrin will require additional investigation.

In addition to its localization to the outer plasma membrane, a surprising finding of this study is that β4 is coex-

pressed with MAG in Schmidt-Lanterman clefts. (This local-
ization is based on immunofluorescent staining as the
Schmidt-Lanterman clefts were not well preserved after fixa-
tion for immunoelectron microscopy.) The presence of MAG
in the Schmidt-Lanterman clefts was previously described
both in vivo (Trapp and Quarles, 1982) and in vitro (Owens
and Bunge, 1989). $\beta4$ is therefore the second membrane pro-
tein to be specifically localized to these structures. This
staining is likely to reflect intact $\beta4$ present in the Schmidt-
Lanterman clefts given that most of the $\beta4$ in the myelinated
cultures is full length with the exception of a small amount
of $\beta4$ that appears to be cleaved in the extracellular domain.
It is not clear what the ligand for $\beta4$ would be in this location
as extracellular matrix components are not known to be pres-
ent within the cleft. Schmidt-Lanterman clefts also contain
F actin and spectrin (Trapp et al., 1989) which have been
proposed, based on this localization, to interact with MAG.
It is not known whether intermediate filaments of Schwann
cells may similarly be concentrated in these sites.

The studies reported here have focused on the expression
of $\alpha6\beta4$ by Schwann cells. $\alpha6\beta4$ is also known to be ex-
pressed by perineurial cells of peripheral nerve (Hogervorst
et al., 1993; Jaakkola et al., 1993). Perineurial cells form
a circumferential cellular sheath around peripheral nerve
fascicles and are believed to be of fibroblastic origin (Bunge
et al., 1989). These cells are in contact with the endoneurial
connective tissue but do not contact either the Schwann cell
or the axon. Thus, induction of $\beta4$ expression in perineurial
cells is likely to be regulated differently than it is in Schwann
cells. Although we have not specifically investigated the ex-
pression of $\beta4$ in fibroblasts, it is our impression that in the
occasional culture in which fibroblasts persist at high levels,
$\beta4$ is expressed at modest levels by cells that form a cellular
sheath around nerve fascicles (and may be an in vitro equiva-
lent to the perineurial sheath). This coculture system may
therefore permit analysis of the factors regulating the expres-
sion of $\beta4$ in perineurial cells. It is also important to note
that fibroblasts contribute to the deposition of the Schwann
cell basal lamina and have important effects on Schwann cell
function in peripheral nerves (Peterson, 1985; Obremski et
al., 1993). Whether they also influence the expression of
integrins by Schwann cells, including the localization and
distribution of $\alpha6\beta4$ is not yet known, but can also be ad-
dressed in this culture system.

Finally, while there are many parallels between myelina-
tion in the peripheral and central nervous systems, the re-
quirement for a basal lamina is unique to the Schwann cell.
In the central nervous system, there is no basal lamina as-
sociated with the oligodendrocyte and in vitro studies have
demonstrated that oligodendrocyte myelination proceeds
normally in the absence of a basal lamina (Eldridge et al.,
1989). Consistent with these findings, oligodendrocytes in
vivo do not express detectable levels of $\alpha6\beta4$ (Sonnenberg
et al., 1990; S. Einheber, T. Milner, F. Giancotti, J. Salzer,
unpublished observations). These findings suggest that $\alpha6\beta4$
expression by Schwann cells reflects their requirement for a
basal lamina and does not correlate with the process of my-
elination per se.

In summary, we have shown that integrins on Schwann
cells are differentially regulated by axonal contact, and that
there is a dramatic increase in the expression of $\beta4$ that ac-
companies the onset of myelination. These studies provide

a framework for future investigations that will be focused on
the molecular basis of the requirement of the basal lamina
in Schwann cell function.

References

Baron-Van Evercooren, A., A. Gansmüller, M. Gumpel, N. Baumann, and
H. K. Kleinman. 1986. Schwann cell differentiation in vitro: extracellular
matrix deposition and interaction. *Dev. Neurosci.* 8:182–196.
Bradley, W. G., and M. Jenkison. 1973. Neural abnormalities in the dystrophic
mouse. *J. Neurol. Sci.* 25:249–255.
Brockes, J. P., K. L. Fields, and M. C. Raff. 1979. Studies on cultured rat
Schwann cells. I. Establishment of purified populations from cultures of pe-
ripheral nerve. *Brain Res.* 165:105–118.
Brunden, K. R., and D. T. Brown. 1990. P0 mRNA expression in cultures of
Schwann cells and neurons that lack basal lamina and myelin. *J. Neurosci.
Res.* 27:159–168.
Buck, C. A., and A. F. Horwitz. 1987. Cell surface receptors for extracellular
matrix molecules. *Annu. Rev. Cell Biol.* 3:179–205.
Bunge, M. B., and R. P. Bunge. 1986. Linkage between Schwann cell extracel-
lular matrix production and ensheathment function. *Annu. N.Y. Acad. Sci.*
486:241–247.
Bunge, M. B., A. K. Williams, and P. M. Wood. 1982. Neuron-Schwann cell
interaction in basal lamina formation. *Dev. Biol.* 92:449–460.
Bunge, M. B., P. M. Wood, L. B. Tynan, M. L. Bates, and J. R. Sanes. 1989.
Perineurium originates from fibroblasts: demonstration in vitro with a
retroviral marker. *Science (Wash. DC).* 243:229–231.
Bunge, R. P., M. B. Bunge, and M. Bates. 1989. Movements of the Schwann
cell nucleus implicate progression of the inner (axon-related) Schwann cell
process during myelination. *J. Cell Biol.* 109:273–284.
Bunge, R. P., M. B. Bunge, and C. F. Eldridge. 1986. Linkage between axonal
ensheathment and basal lamina production by Schwann cells. *Annu. Rev.
Neurosci.* 9:305–328.
Carey, D. J., C. F. Eldridge, C. J. Cornbrooks, R. Timpl, and R. P. Bunge.
1983. Biosynthesis of type IV collagen by cultured rat Schwann cells. *J. Cell
Biol.* 97:473–479.
Carey, D. J., M. S. Todd, and C. M. Rafferty. 1986. Schwann cell myelination:
induction by exogenous basement membrane-like extracellular matrix. *J.
Cell Biol.* 102:2254–2263.
Clark, M. B., and M. B. Bunge. 1989. Cultured Schwann cells assemble
normal-appearing basal lamina only when they ensheathe axons. *Dev. Biol.*
133:393–404.
Cornbrooks, C. J., D. J. Carey, J. A. McDonald, R. Timpl, and R. P. Bunge.
1983. In vivo and in vitro observations on laminin production by Schwann
cells. *Proc. Natl. Acad. Sci. USA.* 80:3850–3854.
Eldridge, C. F., J. R. Sanes, A. Y. Chiu, R. P. Bunge, and C. J. Cornbrooks.
1986. Basal lamina-associated heparan sulphate proteoglycan in the rat PNS:
characterization and localization using monoclonal antibodies. *J. Neu-
rocytol.* 15:37–51.
Eldridge, C. F., M. B. Bunge, R. P. Bunge, and P. M. Wood. 1987. Differenti-
ation of axon-related Schwann cells in vitro. I. Ascorbic acid regulates basal
lamina assembly and myelin formation. *J. Cell Biol.* 105:1023–1034.
Eldridge, C. F., R. P. Bunge, and M. B. Bunge. 1988. Effects of cis-4-hydroxy-
L-proline, and inhibitor of Schwann cell differentiation, on the secretion of
collagenous and noncollagenous proteins by Schwann cells. *Exp. Cell Res.*
174:491–501.
Eldridge, C. F., M. B. Bunge, and R. P. Bunge. 1989. Differentiation of axon-
related Schwann cells in vitro: II. Control of myelin formation by basal lam-
ina. *J. Neurosci.* 9:625–638.
Feltri, M. L., S. Scherer, L. Wrabetz, H. Vogelbacker, P. Bannerman, M. Shy,
and J. Kamholz. 1992. Expression of $\beta4$ integrin in rat peripheral nerve is
developmentally regulated and axonally modulated during regeneration.
Soc. Neurosci. Abstr. 18:1448.
Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha5\beta1$ fibronec-
tin receptor suppress the transformed phenotype of Chinese hamster ovary
cells. *Cell.* 60:849–859.

Giancotti, F. G., M. A. Stepp, S. Suzuki, E. Engvall, and E. Ruoslahti. 1992. Proteolytic processing of endogenous and recombinant β4 integrin subunit. *J. Cell Biol.* 118:951–959.

Hall, D. E., L. F. Reichardt, E. Crowley, B. Holley, H. Moezzi, A. Sonnenberg, and C. H. Damsky. 1990. The alpha 1/beta 1 and alpha 6/beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin. *J. Cell Biol.* 110:2175–2184.

Hemler, M. E., C. Crouse, and A. Sonnenberg. 1989. Association of the VLA alpha 6 subunit with a novel protein. A possible alternative to the common VLA beta 1 subunit on certain cell lines. *J. Biol. Chem.* 264:6529–6535.

Hieda, Y., Y. Nishizawa, J. Uematsu, and K. Owaribe. 1992. Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* 116:1497–1506.

Hogervorst, F., I. Kuikman, d. B. A. E. von, and A. Sonnenberg. 1990. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:765–770.

Hogervorst, F., L. G. Admiraal, C. Niessen, I. Kuikman, H. Janssen, H. Daams, and A. Sonnenberg. 1993. Biochemical characterization and tissue distribution of the A and B variants of the integrin α6 subunit. *J. Cell Biol.* 121:179–191.

Hsiao, L. L., J. Peltonen, S. Jaakkola, H. Gralnick, and J. Uitto. 1991. Plasticity of integrin expression by nerve-derived connective tissue cells. *J. Clin. Invest.* 87:811–820.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

Jaakkola, S., O. Savunen, T. Halme, J. Uitto, and J. Peltonen. 1993. Basement membranes during development of human nerve: Schwann cells and perineurial cells display marked changes in their expression profiles for laminin subunits and β1 and β4 integrins. *J. Neurocytol.* 22:215–230.

Jessen, K. R., and R. Mirsky. 1991. Schwann cell precursors and their development. *Glia.* 4:185–194.

Jessen, K. R., L. Morgan, H. J. Stewart, and R. Mirsky. 1990. Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. *Development (Cambridge).* 109:91–103.

Jones, J. C., M. A. Kurpakus, H. M. Cooper, and V. Quaranta. 1991. A function for the integrin alpha 6 beta 4 in the hemidesmosome. *Cell Regul.* 2:427–438.

Juliano, R. L., and S. Haskill. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577–585.

Kelly, B. M., C. S. Gillespie, D. L. Sherman, and P. J. Brophy. 1992. Schwann cells of the myelin-forming phenotype express neurofilament protein NF-M. *J. Cell Biol.* 118:397–410.

Kennel, S. J., V. Godfrey, L. Y. Chang, T. K. Lankford, L. J. Foote, and A. Makkinje. 1992. The beta 4 subunit of the integrin family is displayed on a restricted subset of endothelium in mice. *J. Cell Sci.* 101:145–150.

Kleitman, N., P. M. Wood, and R. P. Bunge. 1991. Tissue culture methods for the study of myelination. *Culturing Nerve Cells.* William K. Bradford Publishing Co., Acton, MA. 337–377.

Kramer, R. H., and N. Marks. 1989. Identification of integrin collagen receptors on human melanoma cells. *J. Biol. Chem.* 264:4684–4688.

Lee, E. C., M. M. Lotz, J. G. D. Steele, and A. M. Mercurio. 1992. The integrin α6β4 is a laminin receptor. *J. Cell Biol.* 117:671–678.

Lefcort, F., K. Venstrom, J. A. McDonald, and L. F. Reichardt. 1992. Regulation of expression of fibronectin and its receptor, α5β1, during developing and regeneration of peripheral nerve. *Development.* 116:767–782.

Legan, P. K., J. E. Collins, and D. R. Garrod. 1992. The molecular biology of desmosomes and hemidesmosomes: 'What's in a name?'. *Bioessays.* 14:385–393.

Lemke, G., and M. Chao. 1988. Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. *Development.* 102:499–504.

Madrid, R. E., E. Jaros, M. J. Cullen, and W. G. Bradley. 1975. Genetically determined defect of Schwann cell basement membrane in dystrophic mouse. *Nature (Lond.).* 257:319–321.

Martini, R., and M. Schachner. 1986. Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. *J. Cell Biol.* 103:2439–2448.

Moya, F., M. B. Bunge, and R. P. Bunge. 1980. Schwann cells proliferate but fail to differentiate in defined medium. *Proc. Natl. Acad. Sci. USA.* 77:6902–6906.

Obremski, V. J., M. I. Johnson, and M. B. Bunge. 1993. Fibroblasts are required for Schwann cell basal lamina deposition and ensheathment of unmy-

elinated sympathetic neurites in culture. *J. Neurocytol.* 22:102–117.

Owens, G. C., and R. P. Bunge. 1989. Evidence for an early role for myelin-associated glycoprotein in the process of myelination. *Glia.* 2:119–128.

Pedraza, L., A. B. Frey, B. L. Hempstead, D. R. Colman, and J. L. Salzer. 1991. Differential expression of MAG isoforms during development. *J. Neurosci. Res.* 29:141–148.

Peterson, A. C. 1985. Peripheral nerves in shiverer-dystrophic mouse chimera: evidence that a non-Schwann cell component is required for axon ensheathment *in vivo. J. Neurosci.* 5:1740–1754.

Porter, S., M. B. Clark, L. Glaser, and R. P. Bunge. 1986. Schwann cells stimulated to proliferate in the absence of neurons retain full functional capability. *J. Neurosci.* 6:3070–3078.

Quaranta, V., and J. C. R. Jones. 1991. The internal affairs of an integrin. *Trends in Cell Biol.* 1:2–4.

Reichardt, L. F., and K. J. Tomaselli. 1991. Extracellular matrix molecules and their receptors: functions in neural development. *Annu. Rev. Neurosci.* 14:531.

Roberson, M. D., A. D. Toews, J. F. Goodrum, and P. Morell. 1992. Neurofilament and tubulin mRNA expression in Schwann cells. *J. Neurosci. Res.* 33:156–162.

Rosen, C. L., M. P. Lisanti, and J. L. Salzer. 1992. Expression of unique sets of GPI-linked proteins by different primary neurons in vitro. *J. Cell Biol.* 117:617–627.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science.* 238:491–497.

Salzer, J. L., and R. P. Bunge. 1980. Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *J. Cell Biol.* 84:739–752.

Sonnenberg, A., J. Calafat, H. Janssen, H. Daams, L. M. van der Raajj-Helmer, R. Falcioni, S. J. Kennel, J. D. Aplin, J. Baker, M. Loizidou, and D. Garrod. 1991. Integrin α6/β4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *J. Cell Biol.* 113:907–917.

Sonnenberg, A., C. J. Linders, J. H. Daams, and S. J. Kennel. 1990. The α6β1 (VLA-6) and α6β4 protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.* 96:207–217.

Sonnenberg, A., C. J. Linders, P. W. Modderman, C. H. Damsky, M. Aumailley, and R. Timpl. 1990. Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that alpha 6 beta 1 but not alpha 6 beta 4 functions as a major receptor for fragment E8. *J. Cell Biol.* 110:2145–2155.

Stepp, M. A., S. Spurr-Michaud, A. Tisdale, J. Elwell, and I. K. Gipson. 1990. α6β4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl. Acad. Sci. USA.* 87:8970–8974.

Stirling, C. A. 1975. Abnormalities in Schwann cell sheaths in spinal nerve roots of dystrophic mice. *J. Anat.* 119:169–180.

Suzuki, S., and Y. Naitoh. 1990. Amino acid sequence of a novel integrin β4 subunit and primary expression of the mRNA in epithelial cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:757–763.

Tamura, R. N., C. Rozzo, L. Starr, J. Chambers, L. F. Riechardt, H. M. Cooper, and V. Quaranta. 1990. Epithelial integrin alpha 6 beta 4: complete primary structure of alpha 6 and variant forms of beta 4. *J. Cell Biol.* 111:1593–1604.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Toyota, B., S. Carbonetto, and S. David. 1990. A dual laminin/collagen receptor acts in peripheral nerve regeneration. *Proc. Natl. Acad. Sci. USA.* 87:1319–1322.

Trapp, B. D. 1988. Distribution of the myelin-associated glycoprotein and P0 protein during myelin compaction in quaking mouse peripheral nerve. *J. Cell Biol.* 107:675–685.

Trapp, B. D., and R. H. Quarles. 1982. Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. *J. Cell Biol.* 92:877–882.

Trapp, B. D., S. B. Andrews, A. Wong, M. O'Connell, and J. W. Griffin. 1989. Colocalization of the myelin-associated glycoprotein and the microfilament components, F-actin and spectrin, in Schwann cells of myelinated nerve fibres. *J. Neurocytol.* 18:47–60.

Webster, H. d., J. R. Martin, and M. F. O'Connell. 1973. The relationships between interphase Schwann cells and axons before myelination: a quantitative electron microscopic study. *Dev. Biol.* 32:401–416.

Wood, J. G., and E. L. Engel. 1976. Peripheral nerve glycoproteins and myelin fine structure during development of rat sciatic nerve. *J. Neurocytol.* 5:605–615.