Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron–Schwann cell interactions

K. R. JESSEN, L. MORGAN, H. J. S. STEWART and R. MIRSKY

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

Summary

Immunohistochemical methods are used to investigate in detail the development and regulation of three proteins (217c(Ran-1), A5E3 and GFAP) specifically associated with adult non-myelin-forming Schwann cells in the rat sciatic nerve, from embryo day 15 to maturity. 217c(Ran-1), which is probably the NGF-receptor, and A5E3 are expressed by the majority of cells in the nerve at embryo day 15 and by essentially all cells at embryo day 18. GFAP first appears at embryo day 18; this is an intrinsically programmed developmental event which occurs in cultured Schwann cells even in the absence of serum. Postnatally, the expression of 217c(Ran-1), A5E3 and GFAP is suppressed in cells that form myelin but retained in non-myelin-forming Schwann cells. Mature myelin-forming cells nevertheless maintain the potential to express all three proteins but will only do so if removed from contact with myelinated axons. In neuron-free cultures Schwann cells express all three proteins.

This work, together with our previous observations on N-CAM, shows that removal of a diverse set of surface proteins and a change in intermediate filament expression is one of the major consequences of axon to Schwann cell signalling during myelination in the rat sciatic nerve. Unlike myelin-forming cells, adult non-myelin-forming Schwann cells remain very similar to embryonic and newborn cells with respect to expression of surface proteins, in contrast to the previously established developmental changes that occur in their surface lipids.

Key words: Schwann cells, development, myelination, peripheral nerve, GFAP, NGF-receptor.

Introduction

Some of the most important insights into Schwann cell development in recent years have come from studies on the biochemistry of myelin, analysis of the dependence of myelin integrity on contact with appropriate axons, and from studies of the role of the extracellular matrix in myelination (e.g. see Aguayo et al. 1976; Weinberg and Spencer, 1976; Bray et al. 1981; Brostoff, 1984; Bunge et al. 1986; Sutcliffe, 1987). A broader understanding of peripheral nerve maturation must inevitably include an analysis of other aspects of Schwann cell development: two important areas are the prenatal development of Schwann cells prior to the divergent maturation of these cells, and the molecular properties and development of the Schwann cells associated with mature unmyelinated axons (non-myelin-forming Schwann cells).

The establishment of antigenic markers, which change as cells differentiate, has been extremely important in studies of regulatory mechanisms in other developmental systems and would clearly be an important step towards understanding such mechanisms in embryonic Schwann cell development and subsequent Schwann cell diversification. Although there is considerable information on molecular markers of myelin-forming Schwann cells and of Schwann cells in culture there has, until recently, been no systematic attempt to define a set of antigenic differentiation markers for rat Schwann cell development in vivo in embryonic to adult nerves. In this context, we initially examined the molecular phenotype of adult non-myelin-forming cells and showed that, in the rat, these cells express a range of proteins that are not found on adult myelin-forming Schwann cells, although several of them are expressed by astrocytes. Thus non-myelin-forming Schwann cells characteristically express a GFAP-like intermediate filament protein, the surface proteins A5E3 (130×10^3 M_r) and Ran-2 (140×10^3 M_r) and the adhesion molecules N-CAM and Ll/Ng-CAM (Yen and Fields, 1981; Jessen et al. 1984; Jessen and Mirsky, 1984; Mirsky and Jessen, 1984; Mirsky et al. 1986; Martini and Schachner, 1986; Daniloff et al. 1986). These proteins are not present, or found at much lower levels, in...
myelin-forming Schwann cells. Myelin-forming Schwann cells, conversely, express the proteins P0, myelin basic protein (MBP), P2 and myelin-associated glycoprotein (MAG), none of which have been detected in non-myelin-forming Schwann cells (e.g. Trapp et al. 1981, 1988; for reviews see Brostoff, 1984; Norton and Cammer, 1984; Sutcliffe, 1987). In contrast to this phenotype-specific pattern in protein composition, it is intriguing that both adult Schwann cell forms express many of the same lipids, including galactocerebroside, sulfatide (the O4 antigen), and the O8 and O9 lipid antigens (Jessen et al. 1985; Eccleston et al. 1987; Mirsky et al. 1990).

A study of galactocerebroside regulation has shown that expression of this lipid is regulated both by small axons in unmyelinated fibres and by large axons in myelinated fibres, indicating that both small and large axons can up-regulate Schwann cell associated molecules (Jessen et al. 1985, 1987b).

The possibility of a role for suppressive axon–Schwann cell signals was first raised in studies on N-CAM and L1/Ng-CAM expression (Nieke and Schachner, 1985; Danloft et al. 1986; Mirsky et al. 1986; Jessen et al. 1987a). In the rat sciatic nerve, it was shown unambiguously that in development N-CAM, which is present on all neonatal Schwann cells, is down-regulated on myelin-forming Schwann cells as myelination proceeds. This suppression is axon-dependent and rapidly reversible. Thus, individual myelin-forming Schwann cells re-express surface N-CAM within 24 h of removal from axonal contact (Jessen et al. 1987a).

In the present work and in two related papers, we report further on differentiation markers and their regulation in the rat sciatic nerve. In this paper, we have asked firstly, whether the selective and rapidly reversible down-regulation of N-CAM during myelination is also characteristic of other proteins associated with adult non-myelin-forming Schwann cells, namely A5E3, GFAP and a protein identified by the 217c antibody (Peng et al. 1982), which is related to the antigen(s) Ran-I (Fields and Dammerman, 1985) and which we show here for the first time to be a selective marker for non-myelin-forming cells in adult rats. Recent evidence (Qiao Yan, personal communication) indicates that the antigen recognized by the 217c antibody is the NGF receptor, also recognized by the monoclonal antibody 192-IgG (Yan and Johnson, 1988). Second, we have asked when these markers of adult non-myelin-forming Schwann cells appear in the embryonic development of the nerve. This, together with the accompanying paper on the O4 lipid antigen, represents the first systematic study of changes in Schwann cell antigen expression during a key period of rat peripheral nerve development i.e. embryonic day 15 to birth, during which extensive morphological re-arrangements between Schwann cells and axons occur (Peters and Muir, 1959; Gamble, 1966; Webster et al. 1973; Varon and Bunge, 1978; Webster and Favilla, 1984; Ziskind-Conhaim, 1988). A study on the development of the S-100 protein during this period will be presented elsewhere (Jessen et al. 1989).

In summary, the present results together with our previous report on N-CAM (Jessen et al. 1987a), support the proposition that suppression of expression of a distinct set of Schwann cell proteins is one of the major consequences of axon–Schwann cell signalling during myelination. This down-regulation occurs in parallel with the well-established up-regulation of myelin proteins. The results also show that the protein phenotype of adult non-myelin-forming Schwann cells is surprisingly similar to that of embryonic cells, despite large differences between embryonic and adult nerves in Schwann cell plasma membrane lipids (Jessen et al. 1985; Eccleston et al. 1987) and in the morphological relationship between Schwann cells and axons.

Materials and methods

Teased nerve preparations

Sciatic nerves from 4- to 5-week-old Sprague-Dawley rats were excised and desheathed. Portions of the nerves were then gently teased out on to gelatin-coated microscope slides in a drop of phosphate-buffered saline (PBS) using fine syringe needles as described previously (Jessen and Mirsky, 1984). They were allowed to air dry for several hours before immunofluorescent labelling.

Cell suspensions

Cell suspensions of sciatic nerves were prepared from Sprague-Dawley rats. Embryos, 15 to 9 days old (E 15 to 19), were removed from pregnant rats killed by ether anaesthesia followed by bleeding. Newborn to 10-day-old rats were killed by decapitation and older rats were killed by ether anaesthesia followed by bleeding. The sciatic nerves were excised and, where possible, the epineurial sheath was removed. The tissue was placed in an enzyme mixture containing 2 mg ml⁻¹ collagenase ( Worthington), 1.2 mg ml⁻¹ hyaluronidase (Sigma Chemical Co), 0.3 mg ml⁻¹ trypsin inhibitor from chicken egg white (Sigma Chemical Co) in Dulbecco’s Modified Eagle’s Medium (DMEM) without calcium or magnesium, and was then chopped finely. The tissue was incubated at 37°C and 5 % CO₂ for 2 3/4 h (30 day or older rats) 45 min (5 or 10 day rats), or 30 min (E 15–19 or newborn rats). An equal volume of Minimal Eagle’s Medium with 0.02 M Hepes buffer (MEM-H) containing 10 % calf serum was added and the cells gently dissociated through a plastic pipette tip. The cells were centrifuged for 10 min at 500g and resuspended in either MEM-H with 10 % calf serum and antibody if they were to be labelled in suspension, or DMEM supplemented with penicillin (100 U.ml⁻¹), streptomycin (100 mg ml⁻¹), glucose (2 mg ml⁻¹) and glutamine (2 mm) with 10 % fetal calf serum (FCS) if they were to be cultured.

Cell culture

Cell suspensions, prepared as above in DMEM with 10 % FCS, were plated in 30 µl droplets onto glass coverslips, 13 mm in diameter and coated with poly-L-lysine, in a 24-well multiwell plate and kept at 37°C in an incubator gassed with 5 % CO₂/95 % air. After 3 h, the cultures were topped up with 400 µl DMEM with 10 % FCS or changed to serum-free medium, and kept for varying periods of time in the gassed incubator at 37°C. In some experiments, 50 µg ml⁻¹ cycloheximide (Sigma Chemical Co) was added to the cultures at 3 h. The serum-free medium consisted of DMEM and F12 (1:1) supplemented with penicillin, streptomycin, glucose and glu-
tamine as above, and also containing insulin (5 μg ml⁻¹), selenium (0.16 μg ml⁻¹), triiodothyronine (0.1 μg ml⁻¹), transferrin (100 μg ml⁻¹), putrescine (16 μg ml⁻¹), thyroxine (0.4 μg ml⁻¹), progesterone (60 ng ml⁻¹), 30% BSA (ICN Immunobiologicals) (0.3 mg ml⁻¹), dexamethasone (38 ng ml⁻¹).

Derivation
Adult Sprague-Dawley rats weighing 90-110 g were anaesthetised, the left sciatic nerve was cut 2–3 mm below the sciatic notch, and a 2–3 mm segment excised. The rats were then left for 1–2 months, after which they were killed by ether anaesthesia and the distal stump of the left sciatic nerve excised and dissociated as described above.

Antibodies
Monoclonal antibody 217c in the form of culture supernatant was used at a dilution of 1:500. This antibody, first described by Peng et al. (1982), has been shown by Fields and Dammerman (1985) to be equivalent in many respects to the anti-Ran-l serum originally described by Fields et al. (1975) and recognizes an antigen referred to hereafter as 217c(Ran-1). Rabbit antiserum to bovine S-100 protein (Dakopatts A/C) was used at a dilution of 1:400 on dried and fixed preparations, and at 1:800 on cells in dissociated cultures. Monoclonal antibody A5E3, in the form of ascites fluid (Mirsky et al. 1985) was used at a dilution of 1:500. Rabbit antiserum to GFAP (Dakopatts A/C) was used at a dilution of 1:100.

For cultured cells, 217c(Ran-1) and monoclonal antibody to vimentin (clone V9) (Boehringer Mannheim Biochemical) was used at a dilution of 1:50. Monoclonal antibody to laminin, prepared by Dr J. Winter (Winter, 1982) and used at a dilution of 1:50. Monoclonal antibody to vimentin (clone V9) was used at a dilution of 1:500. Rabbit antiserum to laminin, prepared by Dr J. Winter (Lander et al. 1985) was used at a dilution of 1:500. Ascites fluid containing mouse monoclonal antibody to galactocerebroside was produced and characterized by Ranscht et al. (1982). It was used at a dilution of 1:200. Fluorescein conjugated to goat anti-rabbit Ig (G-anti-Rlg-Fl) (Nordic Laboratories Ltd) adsorbed with mouse IgG to remove cross-reacting antibodies, and tetramethyl rhodamine conjugated to goat anti-mouse Ig (G-anti-Mlg-Rd) (Cappel Labs. Inc.), adsorbed with rabbit IgG to remove cross-reacting antibodies, were both used at a dilution of 1:100.

Immunofluorescence
All antibodies were diluted in MEM-H plus 10% calf serum if they were applied to living cells, or in PBS plus 10% calf serum, 0.02% sodium azide and 0.1% lysine if they were applied to dried or fixed cells. All incubations were carried out for 30 min at room temperature except for S-100 on dried preparations (3 h).

Teased nerve preparations
These were allowed to dry for several hours before application of 217c(Ran-1) antibodies, followed by G-anti-Mlg-Rd. Fibres were then fixed with 4% paraformaldehyde in PBS for 10 min prior to mounting.

Cell suspensions
These were immunolabelled with 217c(Ran-1) or A5E3 antibodies by adding the relevant antibody to cells suspended in MEM-H with 10% calf serum. After 30 min cells were washed and incubated with G-anti-Mlg-Rd for 30 min and washed again. They were then dried on to gelatin-coated slides and, if they were to be labelled with only one antibody, fixed for 10 min in 4% paraformaldehyde in PBS. Cells that were to be double labelled after suspension labelling were either rehydrated on the slide for 20 min in 4% paraformaldehyde in PBS, washed and then permeabilized for 10 min in 95% ethanol/5% acetic acid at -20°C (for S-100 antibodies) or directly permeabilized in cold 95% ethanol/5% acetic acid (for GFAP, P0, or MBP antibodies). After further washing cells were sequentially labelled with S-100, GFAP, P0, or MBP antibodies and G-anti-Rlg-Fl. Cell suspensions to be double labelled with GFAP and monoclonal P0 antibodies were dried directly on to microscope slides, permeabilized with ethanol/acetic acid and sequentially labelled with antibodies to GFAP, G-anti-Rlg-Fl, monoclonal P0 antibodies and G-anti-Mlg-Rd.

Cultured cells
These were labelled directly on coverslips using essentially the same methods described for cell suspensions. In some experiments, cells were fixed in cold acid/alcohol and labelled with monoclonal antibodies to vimentin followed by G-anti-Mlg-Rd.

Quantification
All results were based on a minimum of 3 separate experiments. For dried cell suspensions or cultured cells, a minimum of 3 coverslips were counted per experiment and generally 200 to 400 cells were counted per coverslip. Percentage figures represent means and error marks (±) indicate s.e.m.

Results

(1) In adult nerves, 217c(Ran-1), A5E3 and GFAP are undetectable on myelin-forming Schwann cells, but present on non-myelin-forming ones

(A) 217c(Ran-1)
When the sciatic nerve from 4- to 5-week-old rats was teased and treated with the monoclonal antibody 217c(Ran-1), immunolabelling was restricted to the strands of non-myelin-forming Schwann cells (Fig. 1). In contrast, the rat antiserum used originally to define the Ran-1 antigen binds to both types of Schwann cells in situ (Mirsky and Jessen, 1984). To show conclusively that the 217c(Ran-1) antibody was binding to the non-myelin-forming Schwann cells themselves, rather than to the axons they are associated with, cell suspensions, freshly made by enzymic dissociation of 4- to 5-week sciatic nerves, were dried on to microscope slides and double immunolabelled with 217c(Ran-1) and S-100 antibodies to specifically identify Schwann cells. In these experiments, essentially all the S-100* cells that showed the typical morphology of non-myelin-forming cells were also 217c(Ran-1)+ and no 217c(Ran-1) binding was detected on myelin-forming cells, which can be identified morphologically in these preparations. Fig. 2 shows three 217c(Ran-1)+ non-myelin-forming cells. Similar experiments were carried out on cell suspensions from the 4- to 5-week sympathetic trunk, a nerve in which more than 99% of the axons are unmyelin-
Fig. 1. 217c(Ran-1) is restricted to non-myelin-forming Schwann cells in vivo. The pictures show part of at least four myelin-forming Schwann cells. (A) 217c(Ran-1)+ non-myelin-forming cell runs with a bundle of myelin-forming cells and is clearly visible for part of its length (nucleus arrowed in B). (A) Fluorescein optics to visualize 217c(Ran-1); (B) phase contrast. Bar in B 10 μm.

Fig. 2. 217c(Ran-1) on dissociated non-myelin-forming cells. The picture shows three 217c(Ran-1)+ non-myelin-forming cells (nuclei labelled with arrows in A). (A) Fluorescein optics to visualize 217c(Ran-1); (B) phase contrast. Bar in B 10 μm.

Excluding the myelin-forming cells, S-100+ cells of the sympathetic trunk were also 217c(Ran-1)+. The level of 217c(Ran-1) seen on these non-myelin-forming cells was, however, clearly lower than that on cultured Schwann cells.

(B) A5E3 and GFAP

The restriction of A5E3 and GFAP to non-myelin-forming cells (Jessen et al. 1984; Mirsky et al. 1985) was confirmed in experiments in which cell suspensions from adult sciatic nerves were dried on to microscope slides and double immunolabelled with A5E3 and S-100, or A5E3 and GFAP antibodies (Fig. 3).

(II) Prenatal development of 217c(Ran-1) A5E3 and GFAP

In these experiments and many of those below, antibody binding was routinely studied on enzymically dissociated cells which had been in culture for only 2–3 h, or on cells in suspension. This was done to minimize any changes in antigen expression that might occur as a result of removal of the cells from axonal contact or from other endoneurial factors necessary for the maintenance of Schwann cell differentiation. The percentage of cells binding a given antibody in these experiments should therefore mirror closely that obtained in vivo.

The time course of 217c(Ran-1), A5E3 and GFAP appearance is shown in Fig. 4. Briefly, 217c(Ran-1) and A5E3 were present on over 75% of cells at E15–16 and on essentially all cells at E18 and at birth, while GFAP was first detectable at E18 and was found on about half the cells at birth. For comparison with GFAP, we studied another intermediate filament protein, vimentin, which is associated with both non-myelin-forming and myelin-forming Schwann cells in adult nerves. Vimentin immunolabelling was present in all cells from E15 onwards (Figs 4, 5).

Immunostaining of GFAP in 2–3 h cultures from newborn nerves is shown in Fig. 6. In some cells, the labelling was strong and filamentous, while in others it was non-filamentous and of variable intensity. In E18–19 cultures, the immunostaining was non-filamentous. Cells with fibroblastic morphology (generally <5% of the total cell number) were always GFAP− (see
Schwann cell protein down-regulation

Fig. 4. Prenatal development of 217c(Ran-l), A5E3 and vimentin (Vim) (embryonic day 15 to the first postnatal day). Figures are expressed as percentage of the total number of cells present. The filled circles represent means with S.E.M. indicated. In every case, a minimum of 3 separate experiments were carried out, a minimum of two coverslips counted per experiment, and generally 200-400 cells counted per coverslip.

Fig. 7. The number of GFAP⁺ Schwann cells detected in suspensions from newborn animals may represent some overestimation of the number of cells expressing the protein at this age in vivo, since GFAP binding rapidly appears in some previously GFAP⁻ cells on removal from axonal contact (see below).

(III) GFAP negative E18 Schwann cells acquire GFAP in neuron-free cultures

To test whether axonal signals or other factors in the endoneurium were necessary for the developmental appearance of GFAP, neuron-free cultures were prepared from E18 sciatic nerves and maintained in 10% FCS or in serum-free medium. After 3 h, 2/3 days and 6 days in culture the cells were double immunolabelled with 217c(Ran-l) to identify the Schwann cells and GFAP antibodies. As shown in Figs 7 and 8, the

Schwann cells rapidly acquired GFAP and this occurred both under serum and serum-free conditions. In one experiment with cells from E15 nerves, over 50% of the 217c(Ran-l)⁺ Schwann cells were GFAP⁺ when observed after 6 days in culture. Thus, at least at the developmental stages tested here, embryonic Schwann cells do not need neuronal or other endoneurial signals to switch on GFAP expression.

(IV) 217c(Ran-l), A5E3 and GFAP are all expressed transiently in developing myelin cells

At birth about 60% of the Schwann cells in the sciatic nerve are galactocerebroside⁺ developing myelin-forming cells (galactocerebroside appears on non-myelin-forming cells in this nerve later in development, coinciding with the morphological development of unmyelinated fibres (Diner, 1965; Jessen et al. 1985). Most of these cells are still Pₒ⁻. Since essentially all of the Schwann cells in newborn nerves express 217c(Ran-l) and A5E3 (above), it is clear that Schwann cells at the

Fig. 6. GFAP is detectable in many Schwann cells at birth. The pictures show six newborn Schwann cells (labelled with 217c(Ran-l) antibodies in B that have been in culture for 2–3 h. One of these (large arrow in C) shows strong, finely filamentous GFAP labelling, while in two others (small arrows in C) it is diffuse and weaker. (A) Fluorescein optics to visualize GFAP; (B) rhodamine optics to visualize 217c(Ran-l); (C) phase contrast. Bar in C 10 μm.
earliest stage of myelination (i.e. galactocerebroside, P0 cells) express both surface proteins. This was not obvious for GFAP, however, since the proportion of GFAP+ Schwann cells at birth is less than 50% (above). To determine whether the GFAP+ cells and the galactocerebroside+ developing myelin-forming cells were separate populations or whether they partly overlapped, cultures from newborn sciatic nerves were double immunostained with galactocerebroside and GFAP antibodies after 3 h in vitro. This showed that 43±8.0% of the galactocerebroside+ cells also expressed GFAP, and that the GFAP+ cells in the cultures were equally divided between the galactocerebroside+ and galactocerebroside− Schwann cell populations, since 53±4.4% of the GFAP+ cells also expressed galactocerebroside.

Fig. 7. GFAP− Schwann cells acquire GFAP with time in neuron-free cultures. The upper row shows flattened, GFAP− (A) 217c(Ran-1)+ (B) Schwann cells from E17-18 nerves after 2–3 h in culture. The lower row shows cells from E17–18 nerves after 6 days in culture. The 217c(Ran-1)+ (E) Schwann cells are elongated and strongly GFAP (D) immunopositive. Note the absence of GFAP from a 217c(Ran-1)− fibroblast arrowed in F. (A,D) Fluorescein optics to visualize GFAP; (B,E) rhodamine optics to visualize 217c(Ran-1); (C,F) phase contrast. Bar in F 10 μm.

Fig. 8. GFAP− Schwann cells acquire GFAP in neuron-free culture with or without serum. The columns show the percentage of Schwann cells from E17–18 nerves, identified by 217c(Ran-1), which also express GFAP at 3 h, 2/3 days and 6 days in culture. The columns represent means with S.E.M. indicated. In every case a minimum of 3 separate experiments were carried out, a minimum of two coverslips counted per experiment, and generally 200–400 cells counted per coverslip.
showed surface galactocerebroside immunolabelling (Fig. 9). Thus, 217c(Ran-1), A5E3 and GFAP are expressed in galactocerebroside+ cells destined to myelinate.

The second stage of the myelination process in Schwann cells is the appearance of the myelin proteins, including P0, in galactocerebroside+ cells and this coincides with morphological myelin formation. We determined whether P0+ cells from neonatal animals still expressed 217c(Ran-1) and GFAP.

For 217c(Ran-1), cells were prepared from newborn, P2 and P3 sciatic nerves, immunostained in suspension for 217c(Ran-1) and, after drying on to microscope slides, double labelled with P0 antiserum to identify those cells that in vivo had started to make myelin. At these times 2±2.0%, 7±1.3% and 48±3.9% of the total cells present were P0+ respectively. A corresponding decrease in 217c(Ran-1)+ cells occurred during this period: of the total number of cells, 99±2.0%, 93±2.1% and 51±9.9% were 217c(Ran-1)+ in newborn, P2 and P3 nerves, respectively. These figures show that the number of 217c(Ran-1)+ cells in the nerve falls rapidly during the early stages of myelin formation. Furthermore, double labelling experiments showed that essentially all of the P0+ cells were still 217c(Ran-1)+ during the first day of life, while at P2 the proportion of P0+ cells still expressing 217c(Ran-1) had dropped to 34±1.8%. The simplest interpretation of these observations is that the 217c(Ran-1) antigen is down-regulated to undetectable levels soon after, but not before, the appearance of P0+. It follows that the proportion of P0+ 217c(Ran-1)+ cells is an indicator of the number of Schwann cells freshly recruited to the pool of P0+ myelin-forming cells.

For GFAP, cell suspensions freshly prepared from P2 sciatic nerves were dried on to microscope slides, permeabilized and double immunolabelled with monoclonal antibodies directed against P0 to detect myelin-forming cells and GFAP antibodies. It was found that 10% of the P0+ cells also showed GFAP (Fig. 10). Therefore GFAP expression in the perinatal sciatic nerve is not restricted to precursors or cells developing into non-myelin-forming cells. Rather, GFAP synthesis starts at E18–19 and spreads through the Schwann cell population irrespective of whether the cells are in the initial stages of development to myelin formation or not. The relatively low number of P0+ GFAP+ cells most probably indicates that, like 217c(Ran-1), GFAP synthesis is suppressed soon after synthesis of myelin proteins commences. It has been reported previously that GFAP is present in human oligodendrocytes in early stages of myelination (Choi and Kim, 1982).

(V) Down-regulation of 217c(Ran-1), A5E3 and GFAP during myelination is axon-dependent and reversible

We have seen that induction to myelinate is accompanied by down-regulation in 217c(Ran-1), A5E3 and GFAP expression. In adult nerves, these antigens are undetectable on myelin-forming Schwann cells, although they remain fully expressed by the non-myelin-forming cells. To test whether the down-regulation of these proteins depends on continuous contact with the myelinated axons, cultures were prepared from postnatal day 6 or day 10 nerves. The cultures were examined after 3 h, 6 h and 9 h. At each of these time points, the cells were double immunolabelled with P0.
antibodies to identify the myelin-forming cells and with 217c(Ran-1), A5E3 or GFAP antibodies, respectively. The results are shown quantitatively in Fig. 11 and the immunostaining for each antigen separately is illustrated in Figs 12–14. Briefly, in culture all three antigens reappeared quickly in the P₀⁺ cells which in vivo had formed myelin. This return to a developmentally less mature phenotype required fresh protein synthesis but did not depend on the cells re-entering the cell cycle, since less than 1% of the Schwann cells incorporate [³H]thymidine during the first 24 h in cultures of this type (Jessen et al. 1987a). As reported previously, immunohistochemically detectable P₀ gradually disappeared from the cells in these cultures during 3–4 days (e.g. Mirsky et al. 1980). Individual myelin-forming Schwann cells therefore depend acutely on interaction with their axon for down-regulation of 217c(Ran-1), A5E3 and GFAP, as well as for up-regulation of the myelin proteins.

In vivo experiments
We used denervation experiments to confirm that in vivo also, loss of axonal contact results in spread of 217c(Ran-1), A5E3 and GFAP throughout the Schwann cell population. It was found that over 90%, 98±0.8% and 90±5.5% of Schwann cells expressed 217c(Ran-1), A5E3 and GFAP, respectively, in distal stumps of adult nerves transected 4–8 weeks previously.

Discussion
Summary and general comments
It is well established that the dramatic changes in Schwann cell phenotype induced by axons during myelination include up-regulation of expression of several myelin proteins and also of lipids such as galactocerebroside and sulfatide (for references see Introduction). The present work shows that these up-regulatory events are accompanied by another set of myelination-specific events, namely the down-regulation of expression of the surface proteins 217c(Ran-1) and A5E3 and of the intermediate filament protein GFAP. We have previously established that N-CAM is regulated in a similar manner to that described here for 217c(Ran-1), A5E3 and GFAP (Jessen et al. 1987a) and there is also evidence consistent with this type of control of L1/Ng-CAM in mouse, chick and rat Schwann cells (Nieke and Schachner, 1985; Daniloff et al. 1986; Martini and Schachner, 1986, 1988; Mirsky et al. 1986) and preliminary evidence in the same direction for Ran-2 in rat Schwann cells (not shown). Therefore, removal of a diverse set of surface proteins and change in intermediate filament expression is one of the major consequences of axon-Schwann cell signalling during myelination.

We also define a new marker of adult non-myelin-forming Schwann cells, i.e. 217c(Ran-1) and show that 217c(Ran-1) and another marker of adult non-myelin-forming Schwann cells, A5E3, are already present on most Schwann cells as early as E15. GFAP, which in the adult is also selectively expressed by non-myelin-forming cells, appears later in embryonic development and is first seen at E18. GFAP expression is switched on in embryonic Schwann cells in culture, even in the absence of serum or neurons, indicating that GFAP appearance on E18 is an intrinsically programmed developmental event. The expression of two other molecules is known to change rapidly during the period from embryo day 15 to birth. These are the surface lipid 04-sulfatide (Mirsky et al. 1990) and the S-100 protein (Jessen et al. 1989); the regulatory mechanisms in these instances appear to be quite different from that seen with GFAP.

Maturation of neonatal Schwann cells to adult non-myelin-forming cells proceeds without suppression of 217c(Ran-1), A5E3, GFAP or N-CAM. Therefore, their suppression during myelination is an important factor in generating the very different pattern in protein expression observed between the two Schwann cell
types in the adult. It also follows that developmental diversification of Schwann cells, leading to the formation of P_0^+, MAG^+, MBP^+ myelin-forming cells and 217c(Ran-1)^+, A5E3^+, GFAP^+, N-CAM^+ non-myelin-forming cells, is due exclusively to interactions between Schwann cells and the larger axons that induce myelination. Other axonally triggered events in Schwann cell development in the rat include the up-regulation of 04-sulfatide and galactocerebroside. These events occur, however, in all Schwann cells and take place either prior to divergent maturation (04-sulfatide) or in both lines of maturation (galactocerebroside) and therefore do not contribute to molecular diversity between non-myelin-forming and myelin-forming cells.

With respect to adult non-myelin-forming cells, the present work shows that in surface protein composition, as it is presently known, they are remarkably similar to embryonic and newborn cells, in contrast to the marked developmental changes that occur in their surface lipids. Denervation, furthermore, has little effect on protein composition of adult non-myelin-forming cells, although it radically alters the protein composition of myelin-forming cells causing loss of myelin proteins and reappearance of cell surface and intracellular proteins normally restricted to non-myelin-forming cells.

217c(Ran-1) and A5E3
There is considerable evidence that the 217c(Ran-1) antibody (Peng et al. 1982) recognizes an antigen identical to the Ran-1 antigen (Fields and Dammerman, 1985). Ran-1 was originally defined by a mouse antiserum (Fields et al. 1975). Although studies on other systems have shown the Ran-1 antisera and the 217c(Ran-1) antibody bind to a very similar spectrum of the cells, we find that only the antisera (Mirsky and Jessen, 1984) but not the 217c(Ran-1) antibody (present work) binds to mature myelin-forming Schwann cells in the rat although both bind to mature non-myelin-forming cells. The protein recognized by the 217c(Ran-1) antibody is down-regulated several days after galactocerebroside is first expressed but very soon after the appearance of P_0. It seems most likely that the Ran-1 antisera detect more than one epitope, one of which,
Fig. 13. Appearance of A5E3 in myelin-forming Schwann cells (P0+) after removal from axonal contact. The pictures show a Schwann cell from a postnatal day 10 nerve after 9 h in culture. It contains P0+ inclusions (B) and shows surface A5E3 immunolabelling (A). (A) Rhodamine optics to visualize A5E3; (B) fluorescein optics to visualize P0; (C) phase contrast. Bar in C 10 µm.

i.e. the one recognized by the 217c(Ran-1), is down-regulated during myelination. Down-regulation of 217c(Ran-1) binding to Schwann cells has previously been observed during myelin formation in culture (Ranscht et al. 1987).

These observations on the 217c antigen are in broad agreement with previous studies on NGF-receptor expression (Taniuchi et al. 1986, 1988; Heumann et al. 1987; DiStefano & Johnson, 1988; Lemke and Chao, 1988; Yan and Johnson, 1988), as would be expected if they represent the same protein (see Introduction). The only significant difference is that 217c can clearly be detected on adult non-myelin-forming Schwann cells using teased nerves and dissociated-dried cell preparations, both of which show these cells to advantage. The level of 217c on adult non-myelin-forming Schwann cells is, however, lower than that seen on Schwann cells in culture.

The 217c(Ran-1) and A5E3 proteins appear very early in Schwann cell development, 86% and 77%, respectively, of the total cell population plated from E15 nerves expressing the antigens when observed after 2–3 h in vitro. The relationship between these two populations of cells has not yet been examined. It is possible that the 217c(Ran-1)+ cells and the A5E3+

Fig. 14. Appearance of GFAP in myelin-forming Schwann cells (P0+) after removal from axonal contact. The pictures show Schwann cells from postnatal day 10 nerves after 20 h in culture. Four P0+ cells are present (B) and all of them are also GFAP+ (A) (two examples labelled with large, white arrows). In addition, two GFAP+ P0− Schwann cells are present (an example is labelled with small white arrow). Note that a flat, fibroblastic cell (black arrow in C) does not bind GFAP antibodies. (A) Rhodamine optics to visualize GFAP; (B) fluorescein optics to visualize P0; (C) phase contrast. Bar in C 10 µm.

cells essentially represent the same population; alternatively, they may be only partly overlapping. For further studies on early nerve development, it will be of interest to determine whether the cells that do not express
217c(Ran-1) or A5E3 belong to early stages of the Schwann cell lineage or whether they are nonneural cells.

Denervation induced spread of expression of proteins normally showing restricted distribution to most of the Schwann cell population, may be important for nerve regeneration. This has been postulated for NGF and NGF receptors (Johnson et al. 1988) and the presence of L1/NG-CAM on denervated Schwann cells is significant in view of the evidence that this protein is particularly important in axon–Schwann cell interaction during axon elongation (Lagenaur and Lemmon, 1987; Bixby et al. 1988; Seilheimer and Schachner, 1988). It remains to be determined whether A5E3 has a specific role in nerve regeneration.

**GFAP**

We first detected GFAP immunoreactivity at E18. This is somewhat earlier than previously reported for the sciatic nerve (Fields and Yen, 1985) but GFAP immunoreactivity associated with nerves in the rat iris is seen at a similar developmental age (Björkland and Dahl, 1985). GFAP immunoreactivity in Schwann cells and enteric glial cells is consistently demonstrated by a large number of polyclonal GFAP antisera (Jessen et al. 1984; Hacker et al. 1985), and by at least one monoclonal GFAP antibody (G-A-5, Boehringer Manheim; KRJ, RM unpublished observation). It has been shown by immunoblotting of nerves and cultured Schwann cells to reside in a protein with the same apparent molecular weight as astrocyte GFAP (Jessen et al. 1984; Fields and Yen, 1985; Fields and McMenamin, 1985).

Furthermore, peptide mapping of CNS and PNS GFAP generate identical patterns of GFAP immunoreactive bands on immunoblots (Fields and Yen, 1985). In spite of this evidence for a close similarity between the CNS and PNS GFAP proteins, they are probably not identical since some monoclonal GFAP antibodies that bind astrocyte GFAP, do not react with Schwann cell GFAP, and only react relatively weakly with enteric glial cells (Jessen et al. 1984; Björkland and Dahl, 1985). It must be emphasized that Schwann cells do not contain the abundance of intermediate filaments characteristic of astrocytes and enteric glial cells. It is therefore not unexpected that GFAP immunolabelling of Schwann cells is significantly weaker than that of the other two cell types. Furthermore, although GFAP labelling of Schwann cells in nerve sections is distinctly filamentous, GFAP binding in cultured Schwann cells, particularly in our 2–3 h cultures, is often non-filamentous. It is possible that assembly of GFAP into filaments is impaired under culture conditions. The GFAP antibodies are clearly not cross-reacting with vimentin since strong vimentin immunolabelling was seen in all sciatic nerve cells from E15 onwards while binding of GFAP antibodies was first detected at E18, and since fibroblastic cells were always GFAP+.

It was found that GFAP-immunoreactive Schwann cells rapidly appeared in neuron-free cultures of initially GFAP-negative cells obtained from E17 and 18 nerves, and that this occurred both in serum-containing and serum-free medium. In one experiment, similar observations were made on cultures prepared from E15 nerves. Immunochemically detectable GFAP expression therefore proceeds without acute requirement for axonal or other endoneurial signals. This is the first identification of a step in Schwann cell development that appears to be regulated by an intrinsic programme rather than by extrinsic signals. In another nerve, the optic nerve in the CNS, the 0–2A progenitor cells, like early Schwann cells, give rise to GFAP+ myelin cells and GFAP+ non-myelin-forming cells (oligodendrocytes and astrocytes, respectively) (Raff, 1989). The regulation of GFAP expression differs however, between early Schwann cells and 0–2A progenitors, since in the latter cell GFAP expression requires triggering by an extrinsic soluble signal, probably related to ciliary derived neurotrophic factor (CNTF) (Hughes and Raff, 1987; Lillien et al. 1988).

Postnatally, in the period P11–P10, GFAP is found in 50–60% of the total Schwann cell population in 2–3 h cultures. This presumably reflects the balance between acquisition of GFAP by developing Schwann cells and the subsequent down regulation only in those cells that form myelin.

**Concluding comments**

During Schwann cell development in the rat sciatic nerve it is now possible to identify several categories of events, which, on the basis of all available evidence, result from or are strongly influenced by axon–Schwann cell signalling (for refs. see Introduction). These are (I) the early up-regulation of sulfatide (the 04 antigen), which is first seen at E16 and which in adult rats is expressed by both non-myelin-forming and myelin-forming cells; (II) the subsequent up-regulation of galactocerebrosides first seen in developing myelin-forming cells at E18 and substantially later in developing non-myelin-forming cells; (III) the early postnatal up-regulation of the myelin proteins, e.g. P0; and (IV) the parallel, reversible down-regulation to undetectable levels of the protein markers of adult non-myelin-forming cells, including 217c(Ran-1), A5E3, GFAP and N-CAM, seen only in developing myelin-forming cells. In addition, NGF receptors are reported to be suppressed during the maturation of both types of Schwann cells in an axon-dependent manner, although on the basis of identity between 217c and the NGF receptor the present results indicate that significant levels are still expressed on adult non-myelin-forming Schwann cells. The molecular nature of the axonal signals that control this complicated sequence of developmental changes remains a challenging question.

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References


Schwann cell protein down-regulation


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