Expression of Specific Sheath Cell Proteins during Peripheral Nerve Growth and Regeneration in Mammals

Hans Werner Müller, Michael J. Ignatius, Donald H. Hangen, and Eric M. Shooter

Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305. Dr. Müller's present address is Neurologisches Institut, Universität Düsseldorf, D-4000 Düsseldorf 1, Federal Republic of Germany.

Abstract. Protein synthesis in the nerve sheath of injured as well as intact mature and developing sciatic nerves from rat and rabbit was investigated by incubating segments of nerve with [^{35}S]methionine in vitro. The composition of labeled proteins under the different conditions of nerve growth was analyzed by two-dimensional gel electrophoresis and fluorography. The expression of six secreted proteins in rat sciatic nerve with the apparent molecular weights of 70,000 (70 kD), 54,000 (54 kD), 51,000 (51 kD), 39,000 (39 kD), 37,000 (37 kD), and 30,000 (30 kD) was of particular interest because of the correlation of their synthesis and secretion with aspects of nerve growth and regeneration. The synthesis of the 37-kD protein was significantly stimulated during both sciatic nerve development as well as regeneration but not in the intact mature nerve. The expression of this protein appears to be regulated by signal(s) from the axon but not the target. The 70-kD protein was exclusively synthesized in response to axotomy, thus confining its role to some aspect(s) of nerve repair. In contrast, the 54- and 51-kD proteins were expressed in the intact mature nerve sheath. Their synthesis and release was rapidly inhibited upon axotomy but returned to normal or higher levels towards the end of sciatic nerve regeneration, suggesting a role in the maintenance of the integrity of the mature (nongrowing) rat nerve. The 39- and 30-kD proteins were only transiently synthesized within the first week after axotomy.

Two proteins with the apparent molecular masses of 70 and 37 kD were synthesized in denervated rabbit sciatic nerve. The similar molecular weights, net charges, and time-courses of induction suggest a homology between these proteins in rabbit and rat, indicating common molecular responses of peripheral nerve sheath cells to axon injury in both mammalian species.

Effective regeneration of interrupted axons in the mammalian peripheral nervous system requires an environment that supports axon elongation. Strong support for the hypothesis that conditions in the glial environment of injured fibers play an important role in successful axonal elongation has come from studies using transplants containing either central or peripheral glia as conduits of axon growth (2, 3, 21, 26, 28). In addition, it has been shown that sheath cells in denervated peripheral nerve stumps secrete diffusible substances that promote neurite outgrowth in cultured sensory and motoneurons (12, 20).

Recently, attempts have been made to identify selective proteins from cells in the peripheral nerve sheath that might be potential candidates for substances facilitating axon growth and maturation by their enhanced synthesis in regenerating compared with mature nerves (13, 22, 25). Skene and Shooter (22) described the increased synthesis of an acidic 37-kD protein in denervated sheath cells of the distal portion of a crushed rat sciatic nerve. This 37-kD protein was secreted into the extracellular space and accumulated in the peripheral nerve during axon regeneration. A protein with the same molecular weight and net charge was synthesized and released in the central nervous system–glial environment of adult rat optic nerve after crush lesions (18, 22) or in rat spinal cord after hemisection (14). During neonatal development of the peripheral nervous system and the central nervous system in rat, a protein with the same isoelectric point and electrophoretic mobility was synthesized in the sciatic and optic nerves (25) as well as in brain and spinal cord (14). However, a significant difference between the posttraumatic expression of the 37-kD protein in the mammalian peripheral and central nervous system is the fact that this protein accumulates in regenerating peripheral nerve but fails to accumulate in the central nervous system (14). Sheath cells in sciatic nerve also responded to denervation by inhibiting the synthesis and release of two other proteins with the apparent molecular weights of 54 and 51 kD (22). In addition several other proteins in rat sciatic nerve whose synthesis is selectively altered in response to axon injury have been described (13), indicating that the pattern of specific changes in protein expression during Wallerian degeneration and nerve regeneration is rather complex.

The present communication describes specific changes in the relative rate of synthesis of selective proteins in cells in

the nerve sheath during development and regeneration of sciatic nerve axons in rat and rabbit. The role of axons and the peripheral target on the regulation of protein synthesis in sheath cells has been examined with particular reference to a set of secreted proteins. These experiments included (*a*) the spatio-temporal separation of axon degeneration from regeneration, (*b*) a comparison of protein expression between the innervated proximal nerve stump and the denervated distal segment, (*c*) the detailed analysis of the spatial distribution of the sites of protein synthesis along the injured sciatic nerve, and (*d*) the separation of the regenerating nerve segment from the peripheral target. Finally, homologies and interspecies differences between two sheath cell proteins in rat and rabbit sciatic nerve are described.

Materials and Methods

Surgery

Adult male Sprague-Dawley rats (180–240 g; Simonsen Laboratories, Gilroy, CA) were anesthetized with sodium pentobarbital (4–6 mg/100 g body weight) and chloral hydrate (10 mg/100 g body weight) administered intraperitoneally. Left sciatic nerves were exposed by a skin incision and blunt dissection through the thigh muscle layers. The nerves were either crushed with a jeweler's forceps, or two double ligations with 4-0 Ethicon suture were applied in close proximity at upper thigh level before transection of the nerve with a fine pair of scissors between the ligations. Both ligated nerve stumps were then separated by sewing them into local muscle tissue. This surgical procedure successfully prevents for many weeks regenerating axons from entering the distal nerve stump (27). Sciatic nerves of the sham-operated right leg of the rats were used as controls. The adult rats were killed in a CO_2 atmosphere before removal of the nerves. Developing sciatic nerves were dissected from 1-, 3-, and 6-wk-old Sprague Dawley rats after decapitation.

Young adult New Zealand White rabbits were anesthesized with an intramuscular injection of ketamine hydrochloride (50–100 mg/kg body weight) followed by chloral hydrate (150 mg/kg) slowly injected into the lateral ear vein. The sciatic nerves of both legs were exposed. The left nerve was either crushed or transected at upper thigh level as described for the rat nerve. The sham-operated nerves on the right were used as controls. Before removal of the sciatic nerves the rabbits were killed by subsequent intravenous injections of ketamine hydrochloride (250 mg) and euthanasia solution T61 (2 ml, Hoechst, Somerville, NJ).

Labeling and Electrophoretic Analysis of Proteins

Sciatic nerve stumps proximal and/or distal to the site of lesion and comparable pieces of sciatic nerve from control animals were removed rapidly and rinsed in ice-cold methionine-free Dulbecco's modified Eagle's medium. The neural tissue was chopped on an ice-cold acrylic block into 1–2-mm segments (or in some experiments into segments >1-mm or 5–6-mm long), transferred into 1 ml of medium containing 100 μCi of [^{35}S]methionine (specific activity ~ 1,000 Ci/mmol; New England Nuclear, Boston, MA), and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 h. Extracellular soluble and secreted proteins released by the nerve tissue were collected in the incubation medium. The medium fractions were centrifuged at 12,000 *g* to remove the tissue. The supernatant was then dialyzed overnight against 100× vol of ammonium acetate at 4°C with one change of the salt solution before lyophilization. In some experiments the tissue pellet from rat sciatic nerve was resuspended in ice-cold Dulbecco's modified Eagle's medium, washed twice, and resuspended in distilled water before homogenization and subsequent lyophilization. Protein samples were stored frozen at −20°C before analysis in two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)[1] according to O'Farrell (15). The first dimension was isoelectric focusing in cylindrical gels (2 mm in diameter) containing 4% acrylamide, 0.21% bisacrylamide, 9 M urea, 3% Nonidet P-40, 4% pH 3.5–10 ampholytes, and 2% pH 4–6 ampholytes (LKB Instruments, Inc., Gaithersburg, MD). Focusing was carried out at 350 V for 15 h. Before transfer of the cylindrical isoelectric focusing gels onto the second dimension gels, the first dimension gels were soaked for 10–20 min in a transfer buffer containing 100 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% (wt/vol) SDS, 10% glycerol, and bromophenol blue as a tracking dye. Second dimension electrophoresis was in SDS polyacrylamide slab gels using the buffer system of Laemmli (10). Separating gels contained 10% acrylamide and 0.27% bisacrylamide. Samples from distal segments of rat sciatic nerve, for loading onto the gels, always contained the same number of trichloracetic acid (TCA)-precipitable counts as the control sample in any given experiment. With distal segments of sciatic nerve from the rabbit, samples were derived from equal lengths of nerve. Gels were stained with Coomassie Brilliant Blue R before autoradiographic image enhancement (Enhance; New England Nuclear), then dried under reduced pressure and exposed to presensitized Kodak XAR-5 x-ray film. Molecular mass standards (Bio-Rad Laboratories, Richmond, CA) used in 2-D PAGE were phosphorylase b (92.5 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD).

[1] *Abbreviations used in this paper:* 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Results

Expression of Soluble and Membrane-bound Proteins in Sheath Cells of Normal and Regenerating Rat Sciatic Nerve

Fig. 1 compares the synthesis of soluble extracellular proteins and cell-associated proteins in a segment of an intact (control) rat sciatic nerve (Fig. 1, *a* and *c*) with that in the distal portion of a sciatic nerve 1 wk after a crush lesion (Fig. 1, *b* and *d*). Because little or no protein synthesis occurs in axons (11) these analyses reveal proteins synthesized in the non-neuronal cells of the nerve sheath (Schwann cells, fibroblasts, endothelial cells, pericytes, mast cells, perineural, and epineural cells) and in any cells that invade the injured nerve. The number of labeled proteins released into the medium (Fig. 1, *a* and *b*) was much smaller than the number of labeled cell-associated proteins including membrane-bound and intracellular soluble proteins (Fig. 1, *c* and *d*). The amount of TCA-precipitable radioactivity in the secreted fraction was 10–20% that in the cell-associated fraction. Between sham-operated control nerves and crushed nerves, marked differences in the radioactive labeling of a small group of specific proteins were observed. The arrows in Fig. 1, *a* and *b* indicate five proteins with the apparent molecular weights of 70, 54, 51, 37, and 30 kD whose relative rates of synthesis changed when the sciatic nerve was crushed. The isoelectric points of these proteins are very similar, ranging between 5.3 and 5.6. The radioactive labeling of the 70-, 37-, and 30-kD proteins increased after nerve injury, whereas the labeling of the 54- and 51-kD proteins was markedly reduced. Since equal amounts of TCA-precipitable radioactivity were analyzed in the control and in crushed distal nerve segments, these changes in relative rates of synthesis can be considered significant. In addition the relative labeling of one protein (of molecular weight 43 kD and isoelectric point 5.1) remained constant between control and injured distal nerve segments, and this protein therefore served as an internal control (14). The pattern of protein labeling was the same in the proximal segment of the injured nerve as in the control nerve shown in Fig. 1 *a* provided a 1–2-mm segment immediately adjacent to the crush site was not included (22). As noted later (see Fig. 6) a slightly enhanced rate of synthesis of the 37-kD protein was observed in a short proximal segment near the site of injury. The same patterns of labeled proteins and the same changes in relative rates of synthesis were observed when shorter (<1 mm) or larger (5–6 mm) segments of nerve were incubated with [^{35}S]methionine, eliminating the possibility that the changes were due to changes in Schwann cell length during Wallerian degeneration (data not shown). Since in vivo labeling, obtained by injecting

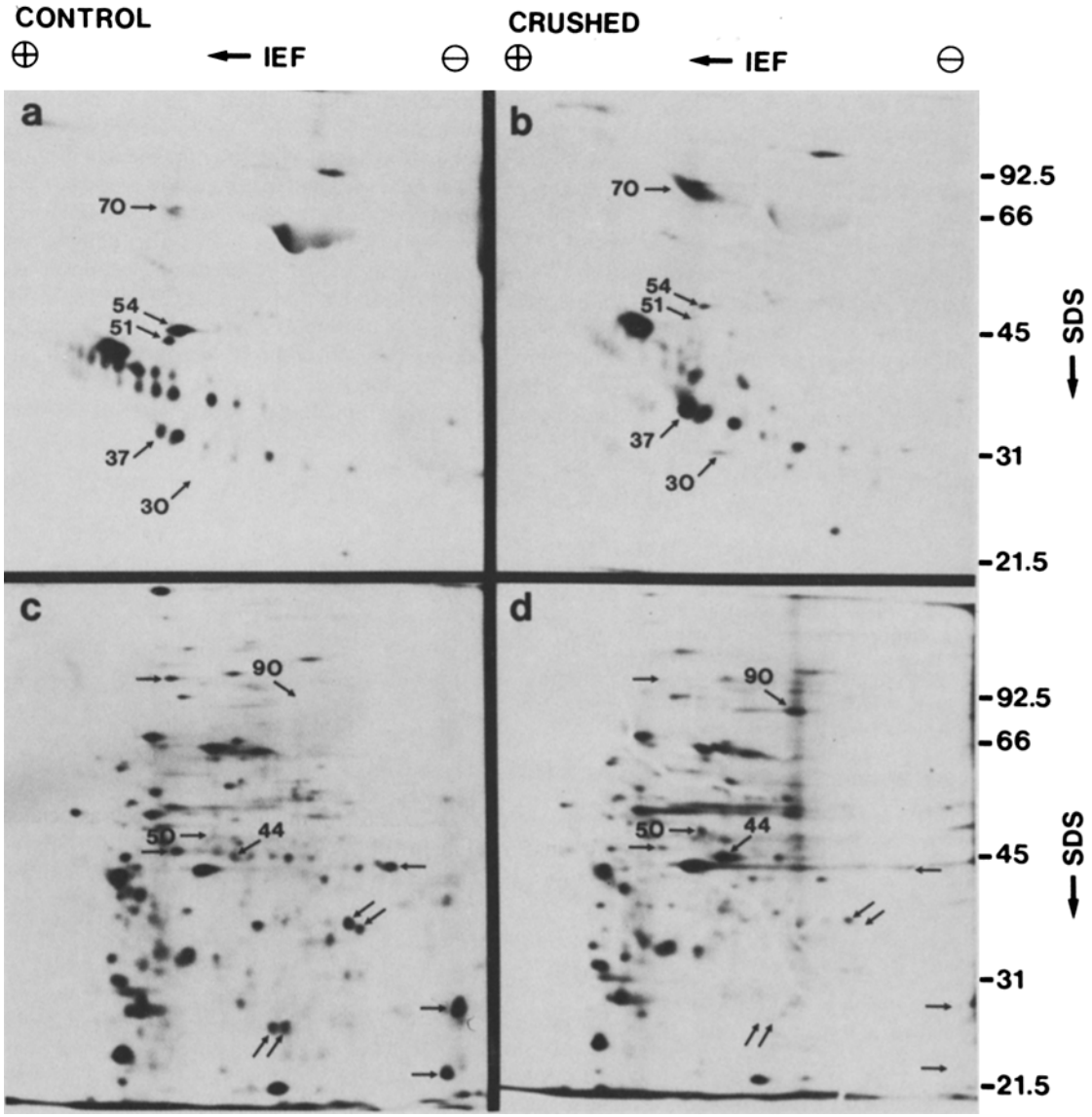

Figure 1. Two-dimensional fluorographs of [^{35}S]methionine-labeled sheath cell proteins from intact and regenerating sciatic nerve of adult rats. (*a*) Soluble extracellular protein fraction from the sciatic nerve of a sham-operated (control) rat and (*b*) from the distal segment of nerve 1 wk after a crush lesion applied at upper thigh level. (*c*) Cell-bound protein fractions from the control sciatic nerve and (*d*) from the crushed nerve. Equal amounts of TCA-precipitable radioactivity were applied on gels *a* and *b*, and *c* and *d*, respectively. The protein samples loaded on gels corresponded to ~1.5 cm of an intact control sciatic nerve (*a* and *c*) or to ~0.5 cm of a crushed nerve (*b* and *d*). The arrows indicate proteins whose labeling reproducibly changed after nerve injury. The numbers to the right of gels (*b* and *d*) indicate the position of molecular weight standards as described in Materials and Methods. Two characteristic proteins appear in these analyses, one (probably actin) of M_r 44,000 and isoelectric point 5.6 and the other of M_r 43,000 and isoelectric point 5.1.

injured sciatic nerve with [^{35}S]methionine, also gave the same pattern of changes in relative rates of synthesis (9), it is clear that the observed changes in in vitro labeling represent physiologically significant processes. The analysis of the pattern of the cell-associated proteins revealed that ~12 proteins reproducibly altered their rate of synthesis in the distal nerve stump after a crush lesion (see arrows in Fig. 1, *c* and *d*). This group of proteins included those polypeptides whose radioactive labeling was either increased (three proteins with molecular masses of 90, 50, and 44 kD) or decreased (nine proteins) as compared with the unaltered pattern of the majority of protein spots. The apparent molecular weight (44 kD) and isoelectric point (pI 5.6) of one of these proteins are similar to those of actin. Nonreproducible changes in the labeling of some other proteins occurred but these are not indicated by arrows in Fig. 1. It seems likely that the changes in labeling represent increases or decreases in rates of protein synthesis rather than changes in rates of protein degradation because the labeling time was relatively short and, for at least one of the proteins, the 37-kD protein, significant extracellular accumulation occurs.

Changes in the Expression of Sheath Cell Proteins during Wallerian Degeneration and Regeneration of Rat Sciatic Nerve

In order to detect specific temporal changes in the expression of sheath cell proteins which are secreted into the vicinity of axons during Wallerian degeneration and nerve regeneration,

we carried out a time-course experiment in which we examined protein synthesis in distal segments of rat sciatic nerves 3, 5, 10, 28, and 56 d after a crush lesion. This time period covers axon and myelin degradation (1–2 wk after crush), sprouting (2 d after crush), axon regrowth in the distal segment (from 3 to 6 d on), and the initiation of regeneration of remyelination as well as Schwann cell proliferation (8). Labeling and accumulation of secreted proteins in the extracellular space of the degenerating and regenerating nerves were compared with intact control nerve (Fig. 2). The rate of synthesis of the 70-kD protein significantly increased in the distal nerve segment within 3 d postcrush and remained at maximal levels for 4 wk before it slowly declined to control values (upper panels). However, the 70-kD protein was not detectable by Coomassie Blue staining (lower panels). On the other hand, the synthesis of the 54- and 51-kD proteins declined to undetectable levels within 3–5 d after a nerve crush but slowly returned to levels even above normal controls within 8 wk postinjury (upper panels). The 54- and 51-kD proteins were also not detectable by Coomassie protein staining (lower panels). Like the 70-kD protein the labeling of the 37-kD protein was increased within 3 d after nerve injury and its rate of synthesis significantly increased to maximal levels within 7–10 d (upper panel). Within 3–4 wk after denervation the 37-kD protein accumulated in the distal portion of the injured nerve up to amounts that represented ~2–5% of the total soluble extracellular proteins as visualized by Coomassie Blue staining (lower panels). Thus the peak of maximal rate of 37-kD protein synthesis preceded the peak of maximal accumulation of this protein in the nerve by ~2 wk. Two

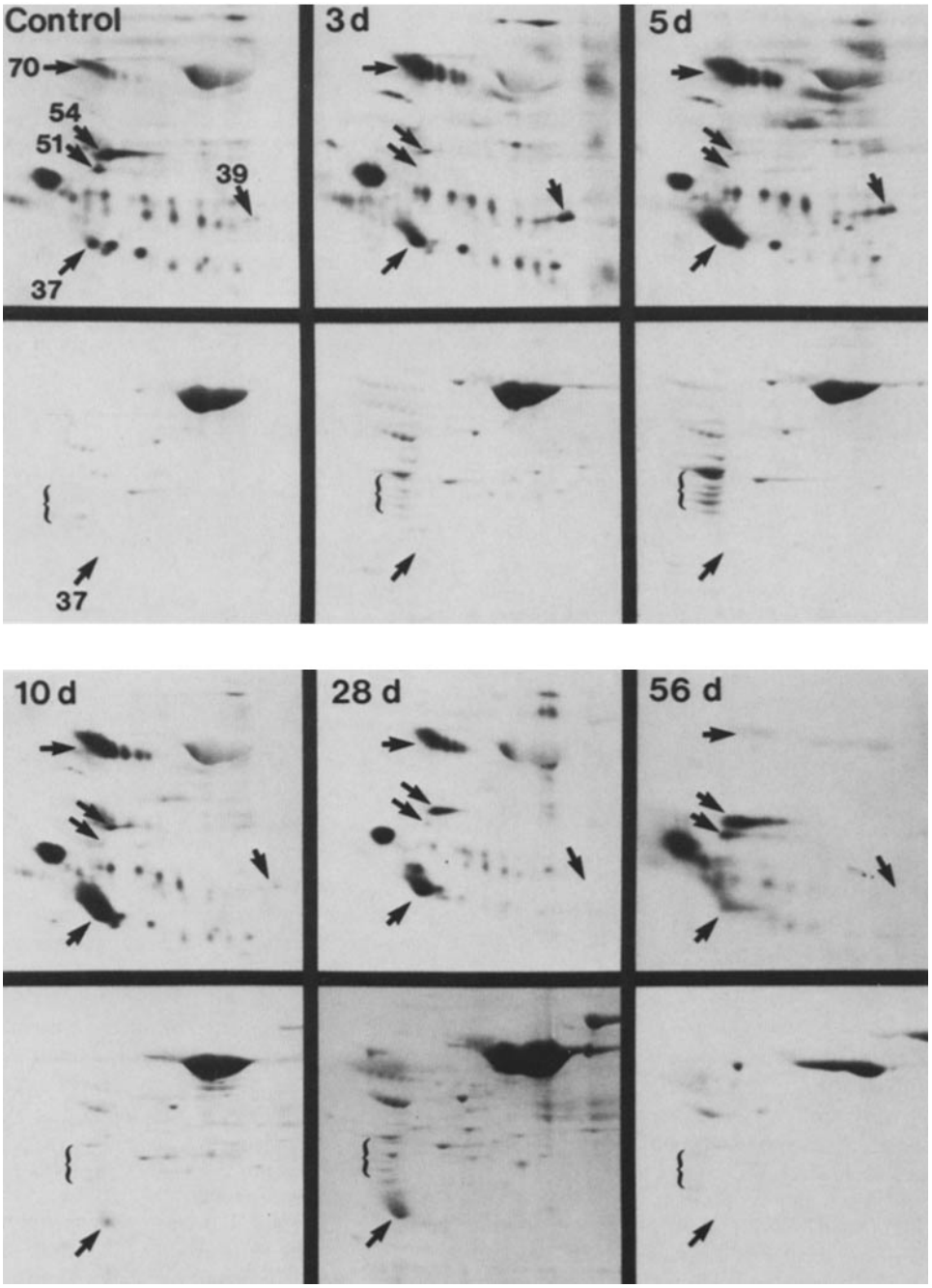

Figure 2. Time-course of the expression of soluble extracellular proteins in sciatic nerve regeneration. The left sciatic nerves of young adult rats were crushed at upper thigh level, and the distal portion of the injured nerves were removed at the times indicated. Extracellular soluble proteins were labeled with [^{35}S]methionine and analyzed by 2-D PAGE as described in Materials and Methods. Equal amounts of TCA-precipitable radioactivity were loaded onto the gels. The gels were first stained with Coomassie Blue before autoradiographic enhancement and fluorography. Sham-operated intact sciatic nerves were processed in the same way and used as controls. Fluorographs of proteins incorporating [^{35}S]methionine are shown in the upper panels. Coomassie Blue stains of the identical gels are shown in the lower panels. Arrows mark the 70-, 54-, 51-, 39-, and 37-kD proteins, respectively. The bracket in the Coomassie Blue–stained gels marks a distinct group of six polypeptides with apparent molecular weights ranging between 45 and 40 kD.

charge isomers of another protein with an apparent molecular weight of 39 kD and an isoelectric point of ~5.9 were transiently synthesized during early periods after axotomy. Although this latter protein was maximally labeled at days 3–5 postlesion (upper panel), it remained undetectable by Coomassie Blue staining (lower panels). A distinct group of six polypeptides with an identical net charge (pI 5.3) and apparent molecular weights ranging from 45 to 40 kD appeared in the distal nerve stump with 3 d postcrush (bracket in lower panels). This group of proteins did not incorporate detectable amounts of [^{35}S]methionine, suggesting either that they do not contain methionine or that they represent degraded unlabeled protein. However, the accumulation of the 45–40 kD proteins upon nerve injury represented a major change in the composition of the soluble extracellular protein pool.

Effect of the Proximal Nerve Segment and the Peripheral Target on the Synthesis of Sheath Cell Proteins in the Denervated Distal Segment of Rat Sciatic Nerve

The role of regenerating axons from the proximal nerve segment and the effect of the peripheral target on the regulation of synthesis and secretion of sheath cell proteins in the distal segment of rat sciatic nerve was investigated by applying various types of surgical lesions to the nerve. In one group of experiments the left sciatic nerve of rats was damaged at upper thigh level by single lesions such as a crush or a transection. After transection the proximal nerve stump was cut back for ~1 cm, and both stumps were tightly ligated. In contrast to a crush lesion the transection of a peripheral nerve and the permanent separation of the two stumps leads to chronic denervation of the distal nerve segment since axons, e.g., from the proximal stump, are prevented from growing into the distal segment. In a second group of experiments sciatic nerves were injured by two lesions simultaneously. One was applied proximal at hip level and the other further distally at the branch point of the tibial nerve. The following combined lesions were carried out: a double-crush, a proximal crush, and a distal transection and a double-transection leading to an isolated segment of sciatic nerve as indicated in Fig. 3. In the latter two types of injury the peripheral target was permanently separated from the nerve segment in between the two lesions. The nerve segments were removed at 1 wk and at 8 wk postinjury and the synthesis of secreted proteins followed in the usual way.

As shown in Fig. 3 (upper panels), the synthesis of the 70- and 37-kD proteins in rat sciatic nerve was increased significantly in all injured nerve segments within 1 wk. Similarly, the synthesis of the 54- and 51-kD proteins was inhibited in all injured nerve segments. This result indicates that neither the proximal nerve stump (Fig. 3, *b* and *e*) nor the peripheral target (Fig. 3, *d* and *e*) affect the changes in synthesis of these proteins in sheath cells. In sciatic nerves where the lesion allowed axon regeneration from the proximal into the distal nerve stump, the high rate of 37-kD synthesis declined to near control rates within 8 wk (Fig. 3, *a*, *c*, and *d*; lower panels). However, in transected sciatic nerves where the elongation of

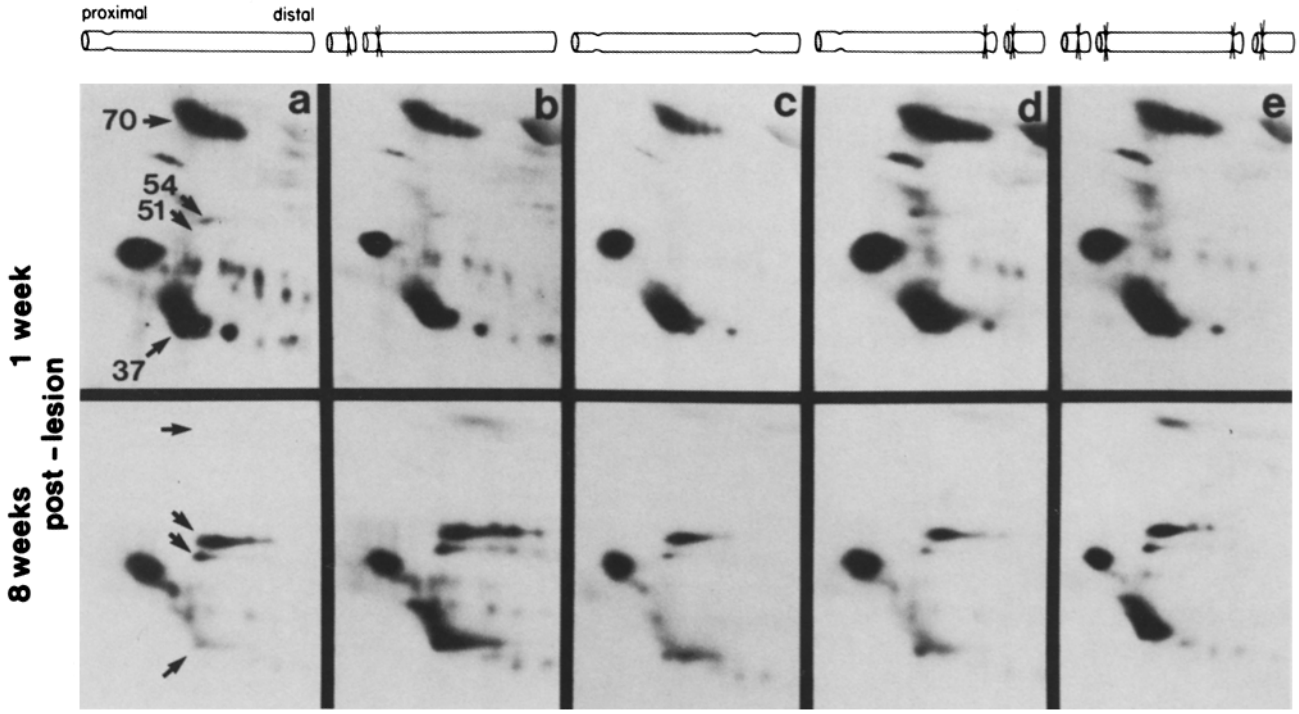

Figure 3. Fluorographs demonstrating the effect of the proximal nerve stump and the peripheral target on the regulation of synthesis of secreted sheath cell proteins in the denervated distal segment of rat sciatic nerve. The left sciatic nerves of young adult rats were injured by the following types of single or double lesions as schematically represented at the top of the figure. (*a*) Crush at upper thigh level; (*b*) transection at upper thigh level and ligation of the two nerve stumps; (*c*) double-crush at hip level and more distally at the branch point of the tibial nerve, respectively; (*d*) crush (proximal) and transection (distal) at the same positions of sciatic nerve as in *c*; (*e*) double-transection and ligation of the isolated nerve segment that was left in place. The lesions were carried out as described in Materials and Methods. 1 and 8 wk after injury the nerves were removed, proteins were metabolically labeled by incubation with [^{35}S]methionine, and soluble extracellular proteins were collected and equal amounts of TCA-precipitable radioactivity were analyzed by 2-D PAGE as described in Materials and Methods.

axons was prevented, the rate of synthesis of the 37-kD protein did not return to the control levels but remained at almost maximal rates for at least 8 wk postinjury (Fig. 3, *b* and *e*; lower panels). Furthermore, isolation of the nerve from the peripheral target had no effect on the maintenance of the rate of 37-kD protein synthesis (Fig. 3*d*). These results suggest that regenerating axons from the proximal nerve stump may provide a signal that inhibits the synthesis of the 37-kD protein in sheath cells of the distal stump. However, the rates of synthesis of the other soluble extracellular proteins such as the 70-, 54-, and 51-kD proteins returned to control levels within 8 wk after denervation regardless of the type of lesion applied, suggesting a different mechanism for the regulation of the synthesis of these proteins in peripheral nerve regeneration. This result also suggests that neither the 70- nor the 54- and 51-kD proteins are related to the 37-kD protein.

Expression of Sheath Cell Proteins in Developing Rat Sciatic Nerve

To investigate the possibility that proteins that are expressed in a regenerating peripheral nerve may also be expressed during de novo nerve growth, sciatic nerves were dissected from 1-, 3-, and 6-wk-old rats and the soluble extracellular proteins were labeled with [^{35}S]methionine as described for regenerating nerves (Fig. 4). As shown in Fig. 4, *a* and *b*, the 37-kD protein was synthesized at a similar high rate in developing rat sciatic nerve early after birth as it was during nerve regeneration in adult animals (Fig. 1*b*). The relative rate of synthesis of the 37-kD protein slowly declined during postnatal development to the low levels of mature control nerves in young adult animals (Fig. 4*c*). This result suggests a role for the 37-kD protein in de novo nerve growth in addition to nerve regeneration. The 70-kD protein, which was induced during nerve regeneration (Fig. 1*b*), was synthesized during nerve development at almost the same low levels as in intact mature sciatic nerves (Fig. 4), suggesting that this protein may have a function in some aspect(s) of nerve repair. The 54- and 51-kD proteins, which were expressed in the mature sciatic nerve of a young adult rat (Fig. 4*c*) but not during early periods of axon regeneration (Fig. 1*b*), were also absent in developing nerves for up to 3 wk after birth (Fig. 4, *a* and *b*). These proteins may thus represent useful markers for the intact mature rat sciatic nerve as well as for advanced stages of nerve regeneration.

Expression of Sheath Cell Proteins in Regenerating Sciatic Nerve from Rabbit

The left sciatic nerves of rabbits were crushed and segments distal to the site of lesion were removed 1, 2, 4, and 8 wk postinjury. Comparable segments of sham-operated intact contralateral sciatic nerves were used as controls. Fig. 5 contains analyses of proteins that were synthesized and secreted into the extracellular space by sheath cells in the distal nerve stump. The synthesis of two acidic proteins with the apparent molecular weights of 70 and 37 kD was reproducibly increased in the denervated distal stump within 7 d after axotomy (Fig. 5, upper panels). This increased rate of synthesis of the 70-kD protein was maintained in the regenerating nerve for at least 2 mo. On the other hand, the increase in the 37-kD protein synthesis was slower and reached its maximal rate at ~2 wk after nerve crush. The rate of 37-kD protein synthesis was significantly reduced at 4 wk postinjury and had slowly declined to even lower levels at 8 wk after the lesion.

The 70-kD protein remained undetectable in Coomassie Blue–stained gels throughout the entire experiment, whereas the 37-kD protein accumulated in the distal stump of the regenerating rabbit sciatic nerve comprise up to ~1–2% of the total extracellular protein within 4 wk after the crush-legion (Fig. 5, lower panels). Thus the peak of maximal concentration of the 37-kD protein trailed the peak of maximal protein synthesis (at 2 wk after injury) by ~2 wk, as seen in the rat.

The molecular weight, the net charge, and the time-course of induction of the 70-kD and 37-kD proteins in the regenerating rabbit nerve were similar to those observed for the 70- and 37-kD proteins from rat (see Figs. 1 and 2), suggesting that these proteins may be homologous. Moreover, both rat and rabbit 37-kD proteins showed microheterogeneity. The 37-kD protein from the rabbit nerve consisted of 4–5 distinct polypeptides that differed mainly in charge (Fig. 5). However, the protein from regenerating rat nerve normally did not resolve into such distinct polypeptides (Figs. 1 and 2) but rather showed a trail, suggesting heterogeneity in both charge and size.

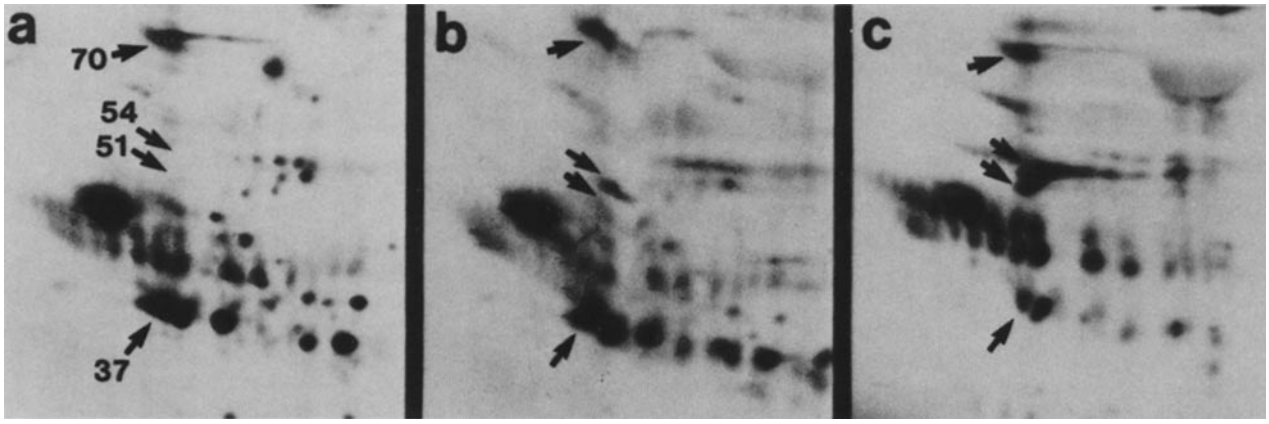

Figure 4. Fluorographs of labeled soluble extracellular proteins from developing rat sciatic nerve. Sciatic nerves were collected from 1 wk (*a*), 3 wk (*b*), and 6 wk (*c*) old rats. The nerves were chopped into 1–2-mm segments, incubated in [^{35}S]methionine, and soluble extracellular proteins collected and separated by 2-D PAGE as described in Materials and Methods. Equal amounts of TCA-precipitable radioactivity were loaded onto gels. The arrows indicate the 70-, 54-, 51-, and 37-kD proteins, respectively.

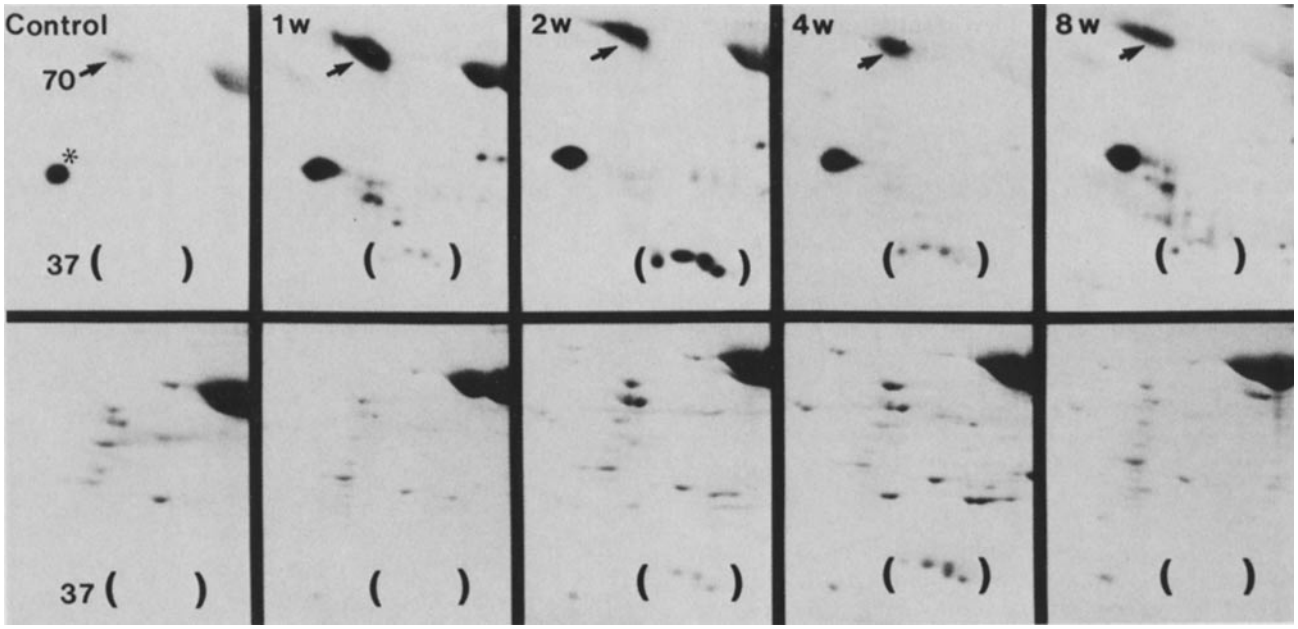

Figure 5. Time-course of the expression of sheath cell proteins in regenerating rabbit sciatic nerve. The left sciatic nerves of young adult rabbits were crushed at upper thigh level and 1-cm segments of nerve from 2–3-cm distal to the site of the lesions were removed 1, 2, 4, and 8 wk postinjury. Each segment of nerve was then further chopped into pieces 1–2-mm long. Proteins in the nerve segments were metabolically labeled with [^{35}S]methionine, and soluble extracellular proteins were collected and analyzed by 2-D PAGE essentially as described in Materials and Methods. Sham-operated intact sciatic nerves were processed in the same way and used as controls. Fluorographs of radioactively labeled proteins as shown in the upper panels. The lower panels represent Coomassie Blue stains of the identical gels. The arrow marks the 70-kD protein, whereas the brackets include the various forms of the 37-kD protein. The asterisk in the fluorograph of the control nerve indicates a 43-kD protein in sciatic nerve medium whose relative labeling does not change upon injury.

Spatial Distribution of the Sites of 37-kD Protein Synthesis in Rabbit Sciatic Nerve after Axotomy

The spatial distribution along the entire rabbit sciatic nerve of the stimulation of 37-kD protein synthesis in response to axon injury was also examined. Sciatic nerves were transected at upper thigh level and the two nerve stumps were ligated and permanently separated to prevent axons from the proximal nerve stump from reentering the denervated distal stump as well as to prevent Schwann cells and other sheath cells of the distal stump from migrating into the proximal segment. The relative rate of [^{35}S]methionine incorporation into the 37-kD protein was estimated from the area and density of the 37-kD protein compared with the 43-kD protein (Fig. 6).

2 wk after injury the 37-kD protein was expressed simultaneously in sheath cells along the entire denervated distal nerve stump from the site of injury to the periphery. However, this protein was synthesized only in a short segment of the proximal stump which was adjacent to the site of injury. The maximal rate of 37-kD synthesis was at least 100-fold above control levels and was observed within 1 or 2 cm distal to the lesion. The rate of synthesis was slower towards the proximal stump. A similar spatial distribution of the 37-kD protein synthesis was observed in crushed sciatic nerves 1 and 4 wk after injury (data not shown). These results demonstrate that the synthesis of the 37-kD protein is stimulated in those segments of an injured rabbit sciatic nerve where axons are known to degenerate (19, 29). The distribution along the rabbit sciatic nerve of the induction of 70-kD protein synthesis in response to axon injury was similar to that of the 37-kD protein (data not shown).

Differential Labeling of the Distinct 37-kD Protein Forms in Regenerating Rabbit Sciatic Nerve

[^{35}S]Methionine incorporation into the different forms of the rabbit 37-kD protein in isolated segments of regenerating sciatic nerve varied along the length of the nerve. At the site of lesion only the more acidic polypeptides of the 37-kD protein were synthesized at a high rate 2 wk after crush (Fig. 7*a*). However, in a nerve segment ~2 cm distal from the crush lesion, the radioactive label was equally distributed between all the 37-kD polypeptides (Fig. 7*b*). In segments further along the distal nerve stump the labeling of the more acidic polypeptides declined, whereas that of the basic polypeptides became more prominent (Fig. 7*c*). A similar shift in radioactive labeling of the 37-kD polypeptides occurred along denervated rabbit sciatic nerve at 1 wk after the injury. However, this variation in radioactive labeling of the 37-kD polypeptides was no longer observed in a sciatic nerve 4 wk after a crush lesion or in a chronically denervated nerve (not shown). These results indicate a differential spatial regulation of the synthesis of the 37-kD protein forms along the distal stump of regenerating rabbit sciatic nerve. In contrast to the 37-kD protein, the rate of synthesis of the charge isomers of the 70-kD protein did not apparently change along the denervated distal nerve stump (Fig. 7).

Discussion

The changes observed in the synthesis of the proteins in rat sciatic nerve sheath after injury as described in the previous section are illustrated in Fig. 8. This study concentrated on

secreted proteins because these are the ones that could immediately affect the environment of the degenerating and regenerating axon. The exact cellular origin of these proteins is not yet known. It is possible that one or more of the resident cells of the nerve sheath (Schwann cells, fibroblasts, etc.) as well as nonresident cells (leukocytes, macrophage) that invade the nerve sheath during Wallerian degeneration (5, 16) may be responsible. In this paper the term sheath cells is used to cover both possibilities. However, it is unlikely, at least for the 37 and 70-kD proteins, that the invading cells account entirely for their enhanced synthesis after nerve injury because the enhanced synthesis is observed over long lengths of the rabbit distal segment within 1 wk after injury. Invasion of an injured nerve by macrophage, for example, is usually restricted to areas around the site of injury (16).

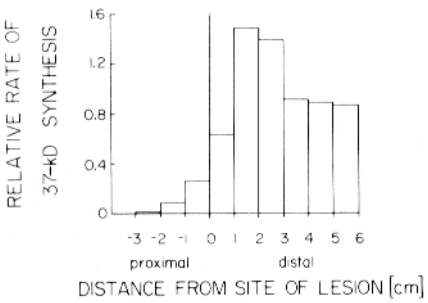

Figure 6. Relative rate of synthesis of the 37-kD protein along a transected rabbit sciatic nerve. The left sciatic nerve of an anesthetized rabbit was exposed and two double ligations with 4-0 suture were applied in close proximity on the nerve at upper thigh level. Then the nerve was transected with a fine pair of scissors between the ligations and both stumps were oriented away from each other and sewed into local muscle tissue. The nerve stumps were reexposed 2 wk after surgery, and 3 cm of the proximal as well as 6 cm of the distal stump were removed and subdivided into 1-cm segments. Each segment was then further chopped into 1–2-mm pieces. Labeling of soluble extracellular proteins in the proximal and distal nerve segments with [^{35}S]methionine and analysis by 2-D PAGE were carried out as described in Materials and Methods. The relative rate of incorporation of radioactive methionine into the 37-kD protein was quantitated by 2-dimensional densitometric scanning of the individual forms of the 37-kD protein on fluorographs and compared with the labeling of an internal standard protein in the nerve medium. The latter protein (marked by an asterisk in Fig. 5, control) has an apparent molecular weight of ~43 kD and a pI of ~5.0. The relative labeling of the 43-kD protein as compared with the total amount of [^{35}S]methionine incorporated into the extracellular soluble proteins of the sciatic nerve remained unchanged after injury. Zero (*0*) indicates the position of the transection in the sciatic nerve. The positive numbers represent 1-cm segments of the distal nerve stump, and the negative numbers represent 1-cm segments of the proximal stump.

The Synthesis of 37-kD Protein in Rat Sciatic Nerve

The enhanced synthesis of the 37-kD protein is observed during both neonatal development and axon regeneration, suggesting a possible function of this protein in nerve growth and maturation rather than with general cellular responses to trauma. The early rise in the synthesis of the 37-kD protein, ~2–3 d after axotomy, coincides with the onset of Schwann cell multiplication (8). However, it is unlikely that the synthesis of the 37-kD protein is related to glial cell proliferation because 37-kD protein synthesis continues in permanently denervated distal stumps (see Fig. 3) at a time when proliferation of Schwann cells is known to have ceased (27). The synthesis of the 37-kD protein is probably not related, either, to the number of Schwann cells because in regenerating nerve segments where Schwann cell numbers remain high (1) its rate of synthesis falls significantly. On the other hand, the rate of synthesis of the 37-kD protein appears to be regulated by axons. This conclusion is drawn from experiments demonstrating that (*a*) the synthesis of the 37-kD protein in the denervated distal stump of an injured nerve declines to the low control levels only under conditions that allow axon regrowth but not under the condition of permanent denervation, and (*b*) the synthesis of the 37-kD protein is enhanced only in those segments of an injured sciatic nerve where axons degenerate.

It is reasonable to assume that the disruption of the intimate axon–Schwann cell contact due to axon degeneration may play a significant role in (re-)stimulating 37-kD protein synthesis in the distal nerve stump after axotomy. It also appears that the high rate of 37-kD synthesis in the distal nerve sheath persists until new axons have grown into the distal stump, thus providing a signal to inhibit 37-kD protein synthesis. The expression of the 37-kD protein during the first 3–6 wk of postnatal development of rat sciatic nerve also indicates that 37-kD synthesis is inhibited when a certain degree of axon maturation is established. As shown in the present study, regulation of the synthesis of the 37-kD protein by the peripheral target is unlikely since its synthesis is not stimulated in nerves separated from the target.

The 37-kD protein has recently been shown to be homologous to apolipoprotein E (9). Since the latter is involved in the transport and metabolism of cholesterol, triglycerides, and phospholipids, it will be of interest to determine if the 37-kD protein plays a similar role with respect to the corresponding lipids in myelin and axonal debris.

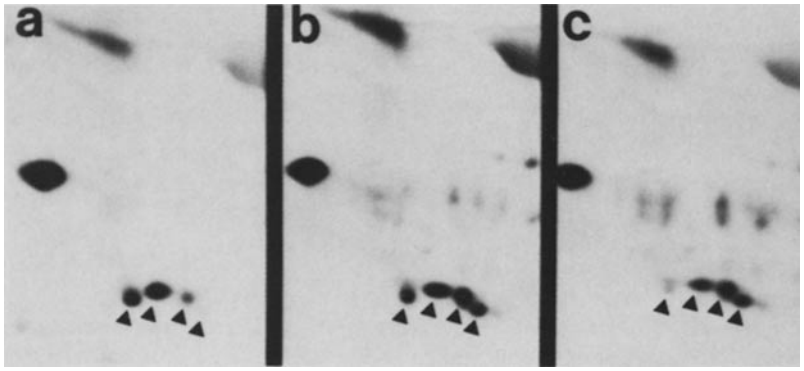

Figure 7. Comparison of [^{35}S]methionine incorporation into the various forms of the 37-kD protein in different segments of regenerating rabbit sciatic nerve. The sciatic nerve was crushed at upper thigh level and the distal nerve stump removed 2 wk after the injury, cut into 1-cm segments, and extracellular soluble proteins in the nerve segments were metabolically labeled with [^{35}S]methionine, collected, and analyzed by 2-D PAGE as described in Materials and Methods. The fluorographs represent segments of the injured nerve: (*a*) at the site of injury, (*b*) 2–3-cm distal from the site of lesion, and (*c*) 4–5-cm distal from the site of lesion. The arrowheads mark the position of the 37-kD polypeptides.

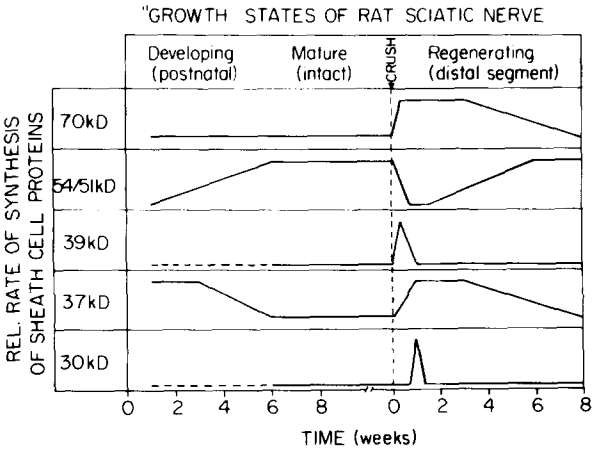

Figure 8. Summary of changes of the relative rates of synthesis of sheath cell proteins in developing, mature, and regenerating rat sciatic nerve.

The Expression of Other Sheath Cell Proteins

The synthesis of the 70-kD protein is enhanced in denervated sheath cells with a similar time-course to that of the 37-kD protein (Fig. 8). However, the experiments with permanently denervated distal stumps, which show that the synthesis of the 70-kD protein declines in the absence of axon regeneration, indicate that the inhibition of the 70-kD protein synthesis is not regulated by axons. Also the role of this protein appears to be confined to some aspect of peripheral nerve repair rather than de novo nerve growth because only the low levels of synthesis characteristic of mature nerve are observed during neonatal development.

The very short periods during which significant synthesis of the low abundant 39- and 30-kD proteins is observed in denervated sheath cells indicate a mechanism of protein induction different from that for the 37- and 70-kD proteins. The transient synthesis of the 39- and 30-kD proteins further suggests that these proteins may be involved in the initiation or termination of specific steps in the temporal sequence of events in nerve regeneration.

The 54- and 51-kD proteins are expressed in the mature intact rat sciatic nerve as well as during advanced stages of nerve regeneration (22). However, the synthesis of these proteins is simultaneously inhibited in both the proximal and distal nerve stumps within 2 d after axotomy. These results suggest that the 54- and 51-kD proteins are candidates either for functions that may be necessary in the intact mature nerve or for functions that normally prevent the transition of the neurons into a growth state. The temporary inhibition of the 54- and 51-kD protein synthesis during an early stage after axotomy may therefore allow the nerve to initiate axon regrowth in the proximal nerve stump. The lack of expression of the 54- and 51-kD proteins in the developing sciatic nerve supports this hypothesis.

When attention is focused on the group of soluble proteins disucssed above, the data indicate that a given 2-D PAGE map of radioactively labeled proteins synthesized and released by rat sciatic nerve sheath cells identifies a particular state of the nerve. Unique protein patterns are obtained for developing, intact-mature, and regenerating nerves as summarized in Fig. 8. In turn this suggests that different physiological states of the sheath cells are represented by distinct programs of gene expression.

Comparison of Sheath Cell Proteins in Rat and Rabbit Sciatic Nerve

The synthesis of two proteins with the apparent molecular weights of 70 and 37 kD are increased in sheath cells of adult rabbit sciatic nerve after axon injury. Besides their molecular weights, these proteins share other properties in common with the corresponding proteins from rat. These include their net charge, the fact that they are microheterogenous, the time-course of the stimulation and inhibition of their synthesis, as well as the spatial distribution of their expression in the injured nerve. These results suggest that the 70- and 37-kD proteins in rat and rabbit may be homologous. If this is so, then the posttraumatic induction of these proteins is part of a specific and common molecular response of a peripheral nerve to injury in these two mammalian species.

The microheterogeneity of the rabbit 37-kD protein is more obvious and distinct than that of the rat protein and it is of interest that the rate of synthesis of the different forms varies along the length of the regenerating rabbit sciatic nerve. This may reflect, at the molecular level, a specific temporal sequence of cellular interactions between the axon and cells in the nerve sheath during peripheral nerve regeneration. It should be noted in this regard that Politis and Spencer (17) were able to separate three distinct morphological zones along a regenerating peripheral nerve in cat which represent successive steps in the maturation of axon–Schwann cell contact.

It is known that neurons respond to axon injury by the induction of specific proteins that are transported along regenerating axons (4, 6, 7, 23, 24). Furthermore, the expression of some of these neuronal proteins is associated with de novo axon growth during development of the central and peripheral nervous system of mammals (24). Combined with these observations, the results described in the present paper support the hypothesis that successful axon growth and maturation requires the sequential expression of specific growth-associated proteins in both sheath cells as well as neurons. Although it is not known how the synthesis of these neuronal and nonneuronal proteins is regulated on the molecular level in a regenerating nerve, the present data suggest that the synthesis of at least one sheath cell protein (the 37-kD protein) is regulated by interactions with axons.

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