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Denervated sheath cells secrete a new protein after nerve injury

(regeneration/glia/Schwann cells/peripheral nerves/central nervous system)

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ABSTRACT **When rat sciatic nerves are crushed, Schwann cells or other supporting cells distal to the injury site begin to synthesize and secrete an acidic 37-kilodalton (kDa) protein. This crush-induced protein accumulates within the nerve sheath and accounts for 2–5% of the total extracellular protein in the distal nerve stump. Synthesis of the 37-kDa protein increases for 2 weeks after nerve crush and declines slowly, beginning 4–6 weeks after the injury. The synthesis of the protein may be regulated by axon–Schwann cell contact. The specific induction of the 37-kDa protein and its accumulation in the extracellular space during nerve regeneration suggest that the protein promotes some aspect of axon growth. Because it is induced slowly after injury, the 37-kDa protein is unlikely to stimulate initial outgrowth of axons; however, it might promote later neuronal responses related to axon growth. The sciatic nerve supporting cells also respond to denervation by reducing the synthesis and release of two proteins of molecular mass 51 and 54 kDa. After crush injury to rat optic nerves, glial cells in the distal optic nerve stump also begin to synthesize and release an acidic 37-kDa protein, although axons of this central nervous system tract do not regenerate. If the 37-kDa protein from peripheral nerves proves to participate in the support of axon regrowth, then the results with rat optic nerve suggest that central nervous system glia initiate at least one part of an appropriate response to nerve injury.**

When peripheral nerves are cut or crushed, the axons distal to the injury degenerate; axons from the proximal nerve stump typically regenerate through the denervated distal nerve stump to reestablish synaptic connections with the periphery. In the central nervous system (CNS) most injured axons fail to regenerate after such injury.

There is considerable evidence that Schwann cells or other supporting cells in the denervated peripheral nerve stump establish an environment that supports axon elongation (1, 2). Schwann cells respond to the loss of axon contact by proliferating and establishing "Büngner bands" through which regenerating axons grow (3, 4). In addition, it has been proposed (4) that denervated nerve stumps secrete a diffusible substance that promotes axon growth. We have been interested in proteins in the distal stumps of regenerating nerves that might act as "signals" to the neuronal cell body for the induction of specific proteins and other activities related to axon growth (5–8) or that support axon growth through local interactions with growing axon tips. In this study the extracellular proteins from the distal stump of a regenerating nerve have been screened in order to select for more detailed study candidates for such signals or growth-promoting proteins.

We reasoned that proteins that stimulate or promote various aspects of axon growth would be unnecessary, and perhaps disruptive to normal functions, in mature nerves. Thus, such proteins might be identified by their specific appearance in the extracellular space of the distal nerve stump during regeneration.

MATERIALS AND METHODS

Surgery. Adult male Sprague–Dawley rats (180–220 g; Simonsen Laboratories, Gilroy, CA) were anesthetized with ketamine hydrochloride (250–500 mg/kg, intraperitoneal). For sciatic nerve crushes, the left sciatic nerve was exposed in the hip and crushed with jeweler's forceps until the nerve was translucent and additional pinches elicited no twitching in leg muscles.

For optic nerve crushes, the left eye was rotated medially, the lateral eye muscles were cut with scissors, and the optic nerve was crushed with jeweler's forceps.

Labeling of Supporting Cell Proteins. At various times, from 2 days to 8 weeks after surgery, rats were killed with a CO_2 atmosphere. Sciatic nerve stumps distal to the crush sites, or comparable pieces of sciatic nerve from control animals or contralateral legs, were removed rapidly and rinsed briefly in ice-cold Dulbecco's modified Eagle's medium (9, 10) without methionine.

Each nerve segment was minced on an ice-cold glass block with a scalpel, and the minced nerve was incubated in 0.5 ml of medium containing 100 μCi of [^{35}S]methionine (1 Ci = 3.7 $\times$ 10^{10} Bq). Incubation was for 3–5 hr at 37°C in a humid atmosphere of 5% CO_2, with vigorous shaking.

Optic nerves were prepared and labeled similarly, using the intracranial portion of the optic nerve. Incubations were in 200 μl of medium.

Sample Preparation. Secreted and extracellular proteins released by the minced nerves during incubation were collected in the incubation medium. The minced tissue was washed with another 0.25 ml of unlabeled medium, and the wash was added to the original medium. The combined medium fraction from each sample was centrifuged 10 min in an Eppendorf microcentrifuge at 4°C to remove cells and cellular debris. The supernatant constituted the "medium" fraction. Proteins were precipitated in 7% trichloroacetic acid and collected by centrifugation at 5,000 $\times$ *g* for 10 min, and the pellets were washed with diethyl ether.

For analyzing intracellular proteins, the minced tissue remaining after the medium had been removed was homogenized in a buffer containing 10 mM Tris·HCl at pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. For determinations of total ^{35}S incorporated into protein, aliquots (usually 10 μl) of each homogenate were added to 1 ml of 10% trichloroacetic acid and filtered through Millipore filters (0.45 μm pore diameter); the filters were washed twice with 1 ml of 10% trichloroacetic acid and dried. Filters were added to 10 ml of scintillation fluid (Econofluor) and radioactivities were measured.

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Abbreviations: CNS, central nervous system; kDa, kilodalton(s); IEF, isoelectric focusing.

For electrophoretic analyses of intracellular proteins, homogenates were centrifuged for 1 hr at 100,000 × g. Pellets constituted a "particulate" fraction. Supernatant proteins ("soluble" fraction) were precipitated by the addition of trichloroacetic acid to 10% and centrifuged 15 min at 10,000 × g, and the pellets were washed with diethyl ether.

Electrophoresis. Protein pellets were dissolved in 0.5% $NaDodSO_4$ containing 2 mM dithiothreitol and heated 2–5 min at 100°C. For determinations of total protein, the dithiothreitol was omitted and total protein was determined by the method of Lowry *et al.* (11). To protein samples dissolved in the $NaDodSO_4$ buffer was added an equal volume of a buffer containing 10% Nonidet P-40, 2 mM dithiothreitol, and 4 M urea.

Samples were analyzed by two-dimensional electrophoresis according to O'Farrell (12). The first dimension was isoelectric focusing in gels (1.5 mm diameter) containing 4% acrylamide, 0.21% bisacrylamide, 9 M urea, 4% pH 3.5–10 ampholytes, and 2% pH 4–6 ampholytes. Focusing was carried out at 350 V for 15 hr. To determine the pH gradient established in the first dimension, duplicate gels were sliced into 1-cm segments; gel segments were soaked overnight in 0.5 ml of distilled water, and the pH was determined.

For transfer to the second dimension of electrophoresis, first-dimension gels were soaked 10–20 min in a transfer buffer containing 100 mM Tris·HCl at pH 7.5, 2 mM dithiothreitol, 2% $NaDodSO_4$, 15% (vol/vol) glycerol, and bromophenol blue as a tracking dye. Second-dimension electrophoresis was in $NaDodSO_4$/polyacrylamide gels in the buffer system of Laemmli (13); running gels contained 10% acrylamide and 0.3% bisacrylamide.

Gels were stained with Coomassie brilliant blue as described by Fairbanks *et al.* (14), dried under reduced pressure, and exposed to Kodak XR-5 x-ray film.

RESULTS

Proteins Secreted by Mature and Crushed Sciatic Nerve. Fig. 1 shows a comparison of the soluble extracellular proteins in mature rat sciatic nerves with those in the distal stump of a sciatic nerve crushed 3 weeks earlier. The soluble extracellular proteins were washed out of the nerves by removing nerve segments from the animals, mincing the nerves, and incubating the minced tissue in a culture medium. Soluble proteins released into the culture medium were analyzed by two-dimensional electrophoresis and stained for protein.

About three times as much protein was recovered in the medium from ≈2-cm lengths of sciatic nerve distal to a crush as from comparable segments of intact nerves. When similar amounts of *total* protein from the medium fractions of control nerve and distal stumps were analyzed the compositions of these fractions were found to be very similar in the two tissues. However, one protein that was a major component of the medium fraction from distal nerve stumps was not detected in the medium fraction from control nerves. This protein migrated with an apparent molecular mass of 37 kDa (37-kDa protein) and an apparent isoelectric point (pI) of 5.4. On the basis of its protein staining, it was estimated that this 37-kDa protein constituted 2–5% of the soluble extracellular protein of the nerve stump distal to the crush site. The 37-kDa protein was first detected between 1 and 2 weeks after sciatic nerve crush in rats.

Induction of 37-kDa Protein Synthesis After Denervation. To determine whether the 37-kDa protein is a product of nonneuronal supporting cells denervated after nerve crush, distal nerve stumps were removed at various times after sciatic nerve crush and incubated briefly (3–5 hr) in culture medium containing [^{35}S]methionine. Because little or no protein synthesis occurs in axons (16) this incubation should label only proteins synthesized by nonneuronal supporting cells of the nerve. Labeled proteins secreted into the medium were analyzed by electrophoresis and detected by autoradiography.

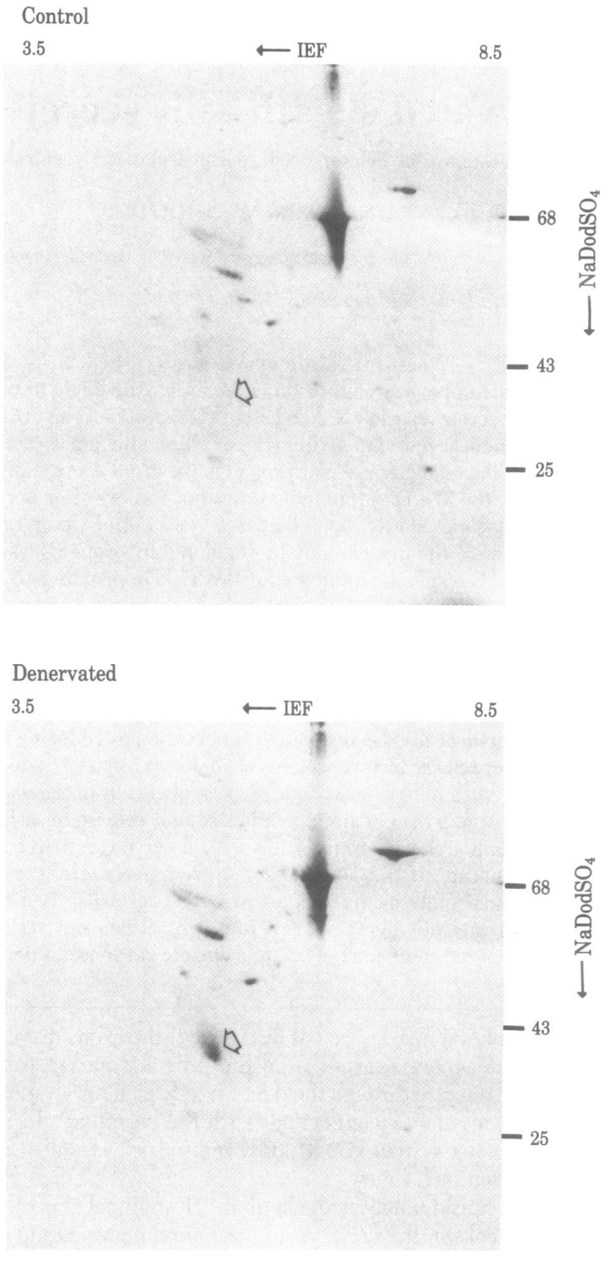

FIG. 1. Coomassie blue-stained gels of extracellular proteins from the distal segment of a rat sciatic nerve 3 weeks after nerve crush (Denervated) and from the undamaged contralateral sciatic nerve (Control). Nerves were minced and incubated 3 hr in culture medium; total protein released into the medium was 0.47 mg for the control nerve and 1.25 mg for the denervated stump. An 85-μg aliquot of total medium fraction protein was loaded onto each gel. The open arrows indicate the 37-kDa protein induced after injury. The numbers to the right of each gel indicate the positions of molecular mass standards: bovine serum albumin (68 kDa); ovalbumin (43 kDa); and chymotrypsinogen *a* (25 kDa). The very heavily stained polypeptide at 68 kDa is probably rat serum albumin (15). IEF, isoelectric focusing.

Fig. 2 shows vignettes representing the portions of gels containing the 37-kDa protein described above. Labeling of the 37-kDa protein was significantly increased between 2 and 7 days after nerve injury; labeling of the protein was maximal by 2 weeks after crush. Between 4 and 6 weeks after crush, labeling

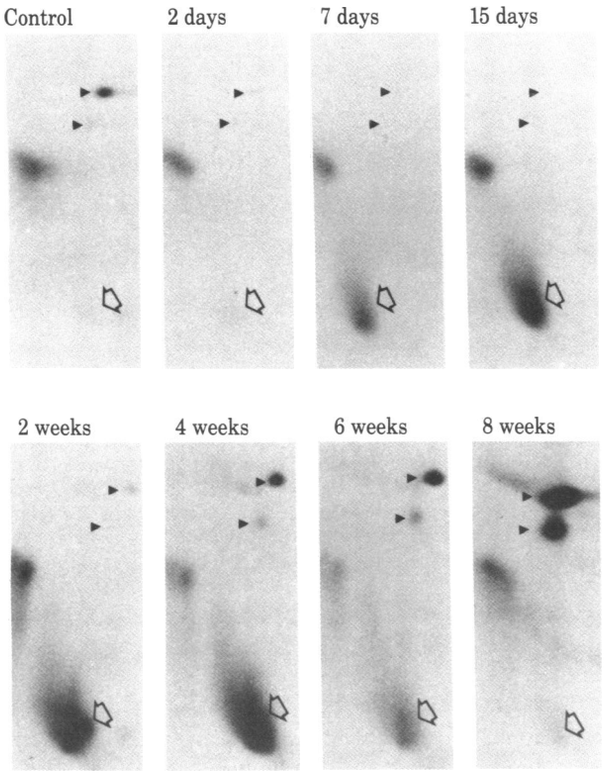

FIG. 2. Changes in synthesis and secretion of protein by nonneuronal cells during sciatic nerve regeneration. At the indicated times after sciatic nerve crushes, the nerve stumps distal to the crush were removed, minced, and incubated with [^{35}S]methionine. Labeled proteins released into the medium were analyzed by two-dimensional gel electrophoresis and autoradiography; the portion of each medium fraction loaded onto a gel represents the same *total* amount of radioactivity incorporated by the nerve stump. The vignettes shown represent regions of the autoradiographs containing the major labeled spots, and include the region containing the Coomassie blue-stained 37-kDa protein shown in Fig. 1. An example of a full autoradiograph is shown in Fig. 3. The control nerve was taken from the leg contralateral to the 15-day crushed nerve; it is typical of both contralateral nerves and sciatic nerves from unoperated animals. The open arrows indicate the 37-kDa protein described in the text; notice that this protein is resolved into two or more components on some gels. The small filled arrowheads indicate the 51-kDa and 54-kDa proteins whose labeling decreases after injury.

of the 37-kDa protein began to decline slowly, and by 8 weeks after crush labeling was far below its peak level but still somewhat above control levels.

Computer-directed densitometric scanning of autoradiographs (J. A. Freeman, personal communication) indicated that incorporation of [^{35}S]methionine into the 37-kDa protein increased 120-fold compared to a control nerve by 15 days after nerve crush. For this comparison, the control nerve autoradiograph containing the heaviest labeling of the 37-kDa protein in any mature nerve was used; labeling of the 37-kDa protein is often undetectable in mature nerve samples. The 120-fold increase is expressed relative to total radioactivity incorporated into protein by the minced nerve; because denervated nerve stumps typically incorporated 2–3 times as much radioactivity into protein as did control nerves, the absolute level of synthesis of the 37-kDa protein in nerve stumps probably increases 250- to 350-fold by 2 weeks after a nerve crush.

To determine whether induction of the 37-kDa protein is confined to the nonneuronal cells that are denervated by the nerve crush, a rat sciatic nerve was crushed at midthigh and 2 weeks later nerve segments approximately 1 cm long were removed from the proximal and distal nerve stumps, avoiding the first 2–3 mm proximal to the crush site. The proximal and distal nerve segments were minced and incubated with [^{35}S]methionine, and labeled proteins released into the medium were analyzed as before. No labeling of the 37-kDa polypeptide in the medium fraction from the proximal nerve segment was detected (data not shown).

Specificity of Induction and Secretion of 37-kDa Protein. Fig. 3 shows labeled proteins released into the medium by a distal nerve stump compared with labeled soluble proteins that remain associated with control nerves or distal stumps after incubation. It is reasonable to assume that these soluble proteins are largely intracellular proteins.

The electrophoretic pattern of labeled proteins in the medium fraction was much simpler than the pattern of proteins remaining associated with the tissue, indicating that the appearance of the 37-kDa protein and other labeled proteins in the incubation medium reflected secretion rather than a nonspecific release of labeled proteins resulting from lysis of cells during the incubation.

There was no detectable labeling of the 37-kDa protein in the presumed intracellular pool from control nerves, indicating that the increased appearance of labeled 37-kDa protein in the medium fraction of distal stumps reflects increased synthesis of the 37-kDa protein rather than increased secretion of a preexisting pool. Finally, there were relatively few differences in the labeling of presumed intracellular proteins between control nerves and distal stumps, indicating that the induction of the 37-kDa protein after denervation is rather specific.

Proteins Decreased After Injury. Fig. 2 also shows that the labeling of two proteins (apparent molecular masses 51 and 54 kDa) decreased after denervation of sciatic nerve supporting cells. The decreased labeling of these proteins and the subsequent recovery of their labeling occurred with a more rapid time course than did the induction of the 37-kDa protein. Labeling of the 51- and 54-kDa proteins declined substantially by 2 days after crush and returned to near the control level by 4 weeks after injury. The increased labeling of the 51- and 54-kDa proteins above the control level late in regeneration, apparent in Fig. 2, was variable. Whether it represents an "overshoot" in the recovery of labeling of these proteins remains to be determined.

Induction of a 37-kDa Protein in Optic Nerve Glia. To compare the response to denervation of nonneuronal supporting cells in a regenerable peripheral nerve with that of supporting cells from a CNS tract that does not regenerate, optic nerves of adult rats were crushed within the orbit. Two weeks later, the cranial portion of the crushed optic nerves was removed, minced, and incubated briefly with [^{35}S]methionine as described for sciatic nerve. The optic nerves contralateral to the crushed nerves were used as controls.

Fig. 4 shows the labeled proteins released into the medium fraction by the nonneuronal cells of the control and denervated optic nerves. Although the overall electrophoretic pattern of proteins released by optic nerve glia did not closely resemble that of proteins released by sciatic nerve supporting cells, a protein with apparent molecular mass 37 kDa and pI approximately 5.5 was more heavily labeled in the denervated sample than in the control.

In addition, the labeling of a second protein (molecular mass $\approx$17 kDa) was also increased in the distal optic nerve stump compared to the control nerve. Coomassie blue staining of these proteins on gels derived from optic nerves has not been observed, but these gels contained less total protein than did gels of sciatic nerve samples.

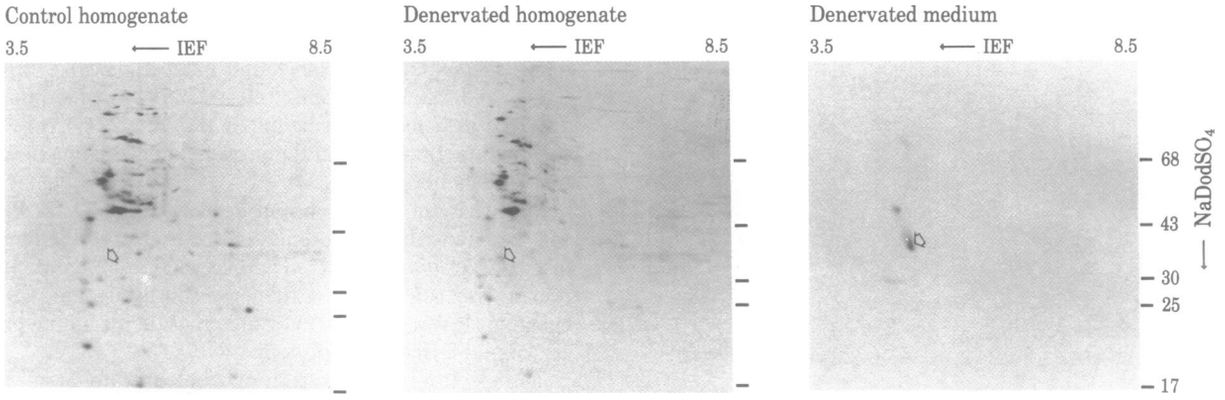

FIG. 3. Comparison of the medium fraction with putative intracellular soluble proteins from control and regenerating rat sciatic nerves. Nerves were incubated with [^{35}S]methionine, the incubation medium was removed, and the tissue was homogenized. The washed tissue was homogenized and centrifuged 1 hr at 100,000 × *g* to remove membrane-bound proteins; the supernatant constituted the "soluble" fraction. (*Left*) Soluble fraction from the sciatic nerve of an unoperated rat. (*Center*) Soluble fraction from the distal stump of a sciatic nerve crushed weeks before labeling. (*Right*) Medium fraction from the distal stump of a sciatic nerve crushed 2 weeks before labeling. The open arrows indicate the position of the 37-kDa protein induced after injury. The numbers to the right indicate the positions of molecular mass standards: bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen *a* (25 kDa), and myoglobin (17 kDa).

DISCUSSION

The data described here indicate that, after injury to a peripheral nerve, nonneuronal cells distal to the injury begin to synthesize and secrete an acidic 37-kDa protein in greatly increased amounts compared to a mature nerve. This 37-kDa protein accumulates extracellularly within the nerve sheath, accounting for 2–5% of the total extracellular soluble protein in the nerve stump. The 37-kDa protein appears to be freely diffusible in the extracellular space, because it can be recovered in the culture medium after incubation of the nerve stump. Alternatively, it could be argued that the nonneuronal cells synthesize and secrete the 37-kDa protein at a constant high level at all times but that contact with axons leads either to efficient uptake or degradation of the secreted protein by the axons. It is unlikely that sequestration of the 37-kDa protein by axons can account for its absence in mature nerve because little, if any, labeled 37-kDa protein was found in intracellular pools of incubated mature nerve. If the phenomenon is to be explained by the action of surface proteases on axons then the turnover of the 37-kDa protein must be extremely rapid in the presence of axons.

The 37-kDa protein is probably synthesized by Schwann cells. The Schwann cells are the most abundant cells in a peripheral nerve stump, and they are most intimately associated with axons, so that they might be expected to be the cells most likely to respond to the degeneration of axons after injury. In addition, Carey and Bunge (17) have shown that rat Schwann cells cultured in the absence of neurons synthesize and secrete proteins with apparent molecular masses of approximately 40 kDa; in collaboration with these authors, we have obtained preliminary evidence that these 40-kDa proteins secreted by cultured Schwann cells include a polypeptide that migrates in two-dimensional electrophoresis with the 37-kDa protein reported here (unpublished data).

The results suggest that interactions between mature axons and Schwann cells chronically repress the synthesis of the 37-kDa protein and stimulate the synthesis of the 51- and 54-kDa proteins. Mature axons might regulate the synthesis of specific Schwann cell proteins through electrical activity (18), intracel-

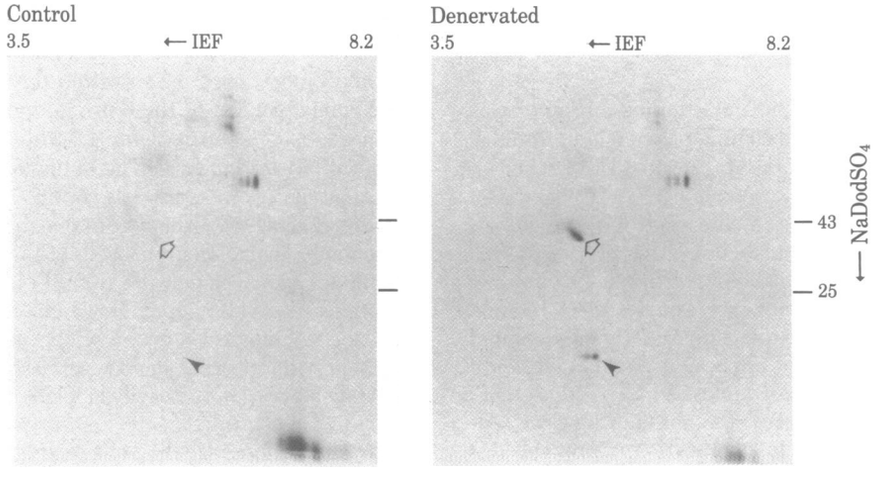

FIG. 4. Induction of a 37-kDa protein in distal stump of a crushed rat optic nerve. The intracranial portions of injured (Denervated) and contralateral (Control) optic nerves were removed 2 weeks after one optic nerve was crushed in the orbit. The nerves were minced and incubated with [^{35}S]methionine, and the labeled proteins released into the medium were analyzed. The open arrows indicate the 37-kDa protein whose labeling increases after injury. The small filled arrowheads indicate a 17-kDa polypeptide whose labeling also increases. The numbers to the right indicate the positions of molecular mass standards: ovalbumin (43 kDa) and chymotrypsinogen *a* (25 kDa).

lular transfer of molecules from axons to Schwann cells (19), or direct contact between axonal and Schwann cell membranes (20). Synthesis of the 51- and 54-kDa proteins decreases within 2 days of a nerve crush, consistent with regulation by electrical activity or a rapidly turning over axonal molecule. Synthesis of the 37-kDa protein is induced slowly over the first 2 weeks after injury, as axons are removed by Wallerian degeneration (4, 5, 21), remains elevated through 4 weeks after crush as fine-caliber axons grow through the distal stump (22–24), and declines slowly as axons mature and reestablish mature axon–Schwann cell contacts (3, 21, 22). This slow time course is consistent with regulation of the 37-kDa protein by contact between Schwann cells and mature axon membranes or by a slowly turning over axonal molecule. It is possible, then, that different aspects of axon–Schwann cell interactions regulate the expression of distinct proteins in the Schwann cells.

The results reported here also indicate that interaction with mature axons regulates the synthesis of an acidic 37-kDa protein by CNS glia. There is no direct evidence that the denervation-induced protein from CNS glia is homologous to the 37-kDa protein induced in peripheral nerve supporting cells. However, the CNS and peripheral nervous system proteins are similar in size, isoelectric point, and solubility; both are secreted by nonneuronal supporting cells and appear to be regulated by axon–support cell interactions. Pellegrino *et al.* (25) have also reported the induction of a 37-kDa protein in rat optic nerves after axonal degeneration. In retinal ganglion cells of lower vertebrates, which do regenerate injured axons, axotomized neurons show increased synthesis of at least one "growth-associated protein" within 2 days of nerve crush, before axons begin to elongate (7); mammalian retinal ganglion cells do not exhibit this response to nerve crush (8). Failure to accomplish this or other early responses to injury may limit the ability of some classes of CNS neurons to respond to the 37-kDa protein or other glial factors favoring axon elongation. A similar possibility was raised by Richardson *et al.* (26).

The specific induction and extracellular accumulation of the 37-kDa protein in response to injury suggests that it may be involved in some aspect(s) of nerve repair. One possibility is that the 37-kDa protein promotes axon elongation or maturation, either through strictly local interaction with growing axons or through retrograde transport of the protein to neuron cell bodies (27, 28). Ramon y Cajal (4) proposed that a diffusible Schwann cell product induced in distal nerve stumps after injury promotes axon elongation. Politis *et al.* (29) and Lundborg *et al.* (30) have provided more recent evidence for influences of distal nerve stumps on axon regeneration *in vivo*; the 37-kDa protein might be involved in some of these influences. Richardson and Ebendal (31) have recently reported that rat sciatic nerve releases two substances that promote neurite outgrowth from chicken embryonic ganglia, the activity of one of them being blocked by antiserum to nerve growth factor. Alternatively, the 37-kDa protein might be involved in maturation or remyelination of new axons. If the 37-kDa protein does affect axon repair, its slow induction over the first 2 weeks after injury suggests that it acts primarily on later stages of growth and maturation.

The appearance of new proteins in the distal stumps of injured nerves has been used here to screen for proteins that might be involved in the support of axon growth. This search has yielded one prominent candidate for further study. It should be possible now to determine whether this protein does indeed exert a favorable influence on axon regeneration in peripheral nerves and whether the induction of an apparently similar protein in injured CNS tracts reflects an appropriate, but insufficient, response of central glia to axon injury.

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