

Accelerated Nerve Regeneration Mediated by Schwann Cells Expressing a Mutant Form of the POU Protein SCIP

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Abstract. After injury, the peripheral nervous system (PNS) is capable of full regeneration and recovery of function. Many molecular events that are the hallmarks of the regenerating PNS are recapitulations of developmental processes. The expression of one such molecule, the POU transcription factor suppressed cAMP-inducible POU protein (SCIP), is required for the establishment of normal nerves and is reexpressed during regeneration. Here we describe markedly accelerated

regeneration and hypertrophy of both myelin and axons in transgenic mice that express an amino-terminal deletion of the SCIP molecule. This mutant SCIP molecule retains the POU-specific and POU homeodomain moieties, which allow for both DNA binding and some protein-protein interaction. We demonstrate that the transgene indirectly effects dramatic axonal changes. This is the first demonstration of a genetically controlled acceleration of neural regeneration.

REGENERATION is a hallmark of the peripheral nervous system (PNS)¹ such that PNS axons are capable of both finding their original target tissues and reestablishing functional synapses with a high degree of fidelity. After compression injury, peripheral nerves undergo a stereotyped pattern of Wallerian degeneration, characterized by myelin decompaction and phagocytosis as well as axonal die-back, which leaves intact endotubes formed by the residual basal lamina and the associated Schwann cells (for review see Griffin et al., 1996). The endotubes form channels into which the regenerated axons will grow. Before degeneration is complete, the PNS begins the process of regeneration that will result in complete recovery. At the onset of axonal regeneration, the proximal nerve stumps form new sprouts that reenter the endotubes (Bray et al., 1972; McQuarrie, 1985), and grow toward their targets. Although the basal lamina is necessary for regeneration, it is not sufficient (Ide et al., 1983; Hall, 1986). Several groups have demonstrated that Schwann cells are associated with and are required for regenerating axons to reenter the distal stump. This is the case whether the fibers are growing into heterologous basal lamina

(Feneley et al., 1991), acellular nerve grafts (Gulati, 1988), or are sprouting into distal nerves after degeneration (Fawcett and Keynes, 1988). In addition, axons fail to regenerate across physical gaps in the absence of Schwann cells (Scaravilli et al., 1986; Jenq et al., 1988; Le Beau et al., 1988). Taken together, these data suggest that viable Schwann cells are required for axonal extension after injury, even in the permissive microenvironment of the basal lamina into which axons readily elongate.

We have been interested in the transcriptional regulation of PNS development and regeneration. The transcription factor suppressed cAMP-inducible POU protein (SCIP) (also known as Oct-6 and Tst-1 [Suzuki et al., 1990; He, 1991]), a member of the POU family of transcription factors, is expressed in both developing and regenerating Schwann cells. SCIP is known to repress the myelin structural genes P_0 and myelin basic protein (MBP) when it is expressed by Schwann cells during a narrow window of development termed promyelination (Monuki et al., 1993; Weinstein et al., 1995). In the adult, SCIP expression is undetectable in the Schwann cell unless the nerve is injured, after which the gene is reexpressed as axons enter the distal nerve stump (Zorick, 1996). We have been interested in the function of SCIP during PNS development and have recently reported on the generation of transgenic mice that express a mutant form of SCIP, termed Δ SCIP (Weinstein et al., 1995). The transgene is under the transcriptional control of the P_0 promoter, which we and others have used to target expression uniquely to the Schwann cell (Lemke et al., 1988; Messing et al., 1992, 1994). The lines of mice we have isolated and described are single-

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1. *Abbreviations used in this paper:* DRG, dorsal root ganglia; GGF, glial growth factor; NGF, nerve growth factor; PNS, peripheral nervous system; rt, room temperature; wt, wild-type; MBP, myelin basic protein; SCIP, suppressed cAMP-inducible POU protein.

copy gene transgenics that express the Δ SCIP transgene in a Schwann cell-specific manner (Weinstein et al., 1995). The mutant SCIP protein has a deleted amino terminus but an intact POU domain, which allows for DNA binding and POU-specific domain protein-protein interactions (Weinstein et al., 1995; Fyodorov and Deneris, 1996). Data from our lab, and from the study of animals that are SCIP-null, suggest that SCIP function is required for the entry into and maintenance of the promyelinating phase of development (Weinstein et al., 1995; Bermingham et al., 1996; Jaegle et al., 1996). Schwann cells from animals that are null at the SCIP locus stall in their differentiation program at the onset of promyelination (Jaegle et al., 1996), and the Δ SCIP animals that express an amino-terminal deleted gene in midpromyelination precociously exit this phase and enter into myelination. This early phenotypic switch results in an alteration in the 1:1 association of myelinating Schwann cells and their axons as well as an overexpression of the myelin structural genes. Based on these data we have proposed a model of PNS development in which SCIP function is required for a normal promyelinating phase, during which the myelin genes are repressed, and the one-to-one relationship of myelinating Schwann cell to axon is established. The end of promyelination is marked by the downregulation of SCIP expression, high levels of myelin gene expression, and the morphologic appearance of myelin around axons.

In many respects, regeneration recapitulates peripheral nerve development in that SCIP is reexpressed after peripheral nerve injury, and the myelin genes are transiently downregulated during Wallerian degeneration. Yet, unlike promyelination, when SCIP is expressed in a tightly restricted manner, SCIP is expressed for extended periods after injury (Scherer et al., 1994). It is difficult to infer the function of this gene product during regeneration from these data, as the expression does not coincide with regenerative changes in the nerve at late stages after crush. The Δ SCIP mice have yielded a great deal of insight into the function of and requirement for SCIP during development. Here we have used these mice to study the *in vivo* function of SCIP during peripheral nerve regeneration.

Materials and Methods

Surgery

For sciatic nerve crush experiments, the mice were anaesthetized and the right flank and leg were shaved and bathed in 70% EtOH. A single incision was made extending from the knee to the dorsal midline. The skin was retracted and the musculature overlying the sciatic nerve was separated and retracted. The sciatic nerve was isolated and then the sciatic notch was located. The nerve was crushed 5 mm below the sciatic notch with a number 5 Dumont forceps by the application of pressure for 30 s. Pressure was released and then the crush was repeated for an additional 30 s. The muscle and the overlying skin were sutured and then the animal was placed into the cage in the left lateral position under warming lights and allowed to recover for the indicated times [$n = 12$ Δ SCIP (Δ SCIP $^{+}$) and 12 wild type (wt) ($+/+$ CB6f1 animals)].

Electron Microscopy

Mice were anaesthetized with *i.p.* injection of 0.5 cc of 2.5% Avertin/saline before perfusing via the left ventricle. The animals were perfused for 30 s at 37°C with rat ringers containing heparin (2.0 ml/L, stock is 10,000 U/ml) and 2% lidocaine (3.0 ml/L), followed by 7–10 min with 2% glu-

taraldehyde/1% paraformaldehyde in 0.15 M sodium cacodylate, pH 7.2. The primary fixation was carried out for 2–4 h total at 4°C, with 2% glutaraldehyde/1% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.2. Tissues were rinsed six times for 20 min each followed by an overnight rinse at 4°C in 0.15 M sodium cacodylate buffer, pH 7.2. Secondary fixation was carried out for 4 h at 4°C in 1% osmium-tetroxide/1.5% potassium-ferrocyanide in 0.15 M sodium cacodylate buffer, pH 7.2. Tissues were rinsed three times for 10 min each in Millipore-filtered water (Millipore Corp., Waters Chromatography, Milford, MA) at 4°C followed by en bloc staining in 2% uranyl acetate (aq) at 4°C for 1 h. At this point the tissues were dehydrated one time for 8 min in a graded ethanol series starting with water, 30, 50, 70, and 95% followed by two times for 10 min each in 100% ethanol and finally propylene oxide. After dehydration, the nerve tissue was infiltrated with propylene oxide/Durcupan (Fluka Chemika-BioChemika, Ronkonkoma, NY), in a 25:75 ratio for 60 min at room temperature (rt). This was followed by three times for 120 min each in Durcupan resin at rt. Sciatic nerves were flat embedded in fresh Durcupan resin and then polymerized for 24–36 h at 65°C. 1- μ m-thick sections were stained in Toluidine blue. Silver sections were cut on a Diatome diamond knife and then stained with 2% uranyl acetate for 30 min at rt and Reynold's lead citrate for 7 min. Thin sections were viewed at 60 kv and then photographed on a conventional transmission electron microscope (model 100 CX; JEOL USA, Inc., Peabody, MA).

Western Blot Analysis

Sciatic nerves were removed from both transgenic animals and nontransgenic littermates. The length of the tissue was carefully assessed and then the protein was extracted overnight at 4°C in 100 mM Tris, pH 6.8, and 1% SDS and delipidated with ice-cold acetone. The total protein yield was assessed using a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL). Samples were solubilized in standard SDS sample buffer, denatured by boiling, loaded onto a 12% SDS-PAGE gel, and then run at a constant 30 mA. The proteins were then transferred to 0.2- μ m nitrocellulose paper (Schleicher and Schuell Inc., Keene, NH) in a semidry blotting apparatus (Hoefer Scientific, San Francisco, CA). The blot was stained with amido black (Sigma Chemical Co., St. Louis, MO) to assess protein transfer and then blocked with a solution of 5% nonfat milk, 5% normal goat serum (NGS) (Gibco Laboratories, Grand Island, NY) in Tris-buffered saline, pH 7.5 (TBS), for 1 h at rt. The blots were then exposed for 1 h at rt to either a polyclonal rabbit anti-P₀ (gift of D. Colman, Mt. Sinai School of Medicine, NY), rabbit anti-MBP (gift of C. Campagnoni, University of California School of Medicine, Los Angeles, CA), mAb anti-connexin 32 (gift of E. Hertzberg, Albert Einstein College of Medicine, Bronx, NY), or TuJ1 (1:1,000, gift of A. Frankfurter, University of Virginia, Charlottesville, VA), and after five washes were exposed to either anti-rabbit or anti-mouse [¹²⁵I]Ig secondary antibody and then autoradiographed. P₀, TuJ1, MBP, and connexin 32 blots were quantitated on a PhosphorImager as described (STORM; Molecular Dynamics, Inc., Sunnyvale, CA).

Morphometric Analysis

Photographic prints of electron micrographs, covering identical areas of control and transgenic sciatic nerve, were scanned into an Adobe Photoshop file (Aldus, San Diego, CA). Montages were assembled that represented full cross-sectional areas across the nerve and then were transferred to NIH Image (National Institutes of Health, Bethesda, MD), an image analysis program. The imaged montages were assessed for cross-sectional areas of axons, for axon-myelin complexes, and for myelin alone, in 1-mm² areas. Data manipulation was carried out using the Microsoft Excel program (Microsoft, Redmond, WA).

Electrophysiology

The studies were carried out essentially as described by us earlier (Bieri et al., 1997). In brief, adult mice expressing the Δ SCIP transgene and wild-type littermate controls were evaluated using peripheral nerve electrophysiological indices ($n = 4$). In each subject, whole nerve measures of maximal conduction were obtained from the distal sural sensory nerve, both ipsi- and contralateral to the crush. Recordings were performed under general anaesthesia using ketamine (50 mg/kg, *i.p.*) and xylazine (40 mg/kg, *i.p.*) in a sound-attenuated and electrically shielded recording chamber. Temperature was maintained using a circulating warm water bath and monitored using rectal and surface probes. Conduction and amplitude measurements were obtained using standard, noninvasive stimulating and recording techniques, as previously described (Maycox et al., 1997).

Cell Culture

Schwann cells were cultured in DME with 10% FCS and 2–4 mM forskolin (Calbiochem-Novabiochem Corp., San Diego, CA), as described previously (Lemke et al., 1988). Cultures from individual animals were maintained separately and each animal was genotyped by PCR with transgene-specific primers spanning the P₀ 5' UTR (5' CGCTCTCTCCACCCACAGAC 3') to the Δ SCIP transgene (5' GCCGCCTCCCGCCGACAGCAT 3'). Individual cultures grown in DME and supplemented with 10% FCS and antibiotics were expanded in the presence of 1 μ M of forskolin and 50 μ g/ml of glial growth factor (GGF). For the dorsal root ganglia (DRG) axonal outgrowth assays, the Schwann cells were split into 60-mm dishes and then cultured in DME supplemented with 1% FCS and 0.5% horse serum without forskolin or GGF for 3 d before the addition of the DRGs. 1 d before the addition of the rat DRGs to the cultures, the cells were switched to DME supplemented with 1% FCS and 0.5% horse serum and 100 ng/ml of recombinant NGF. Brachial and lumbar DRGs were harvested from postnatal day 0 Sprague-Dawley rats, stripped, and then added to the cultures in fresh medium with NGF (100 ng/ml). Cultures were allowed to grow an additional 20 h and were then fixed in 4% paraformaldehyde. The cells were permeabilized and blocked in 10% NGS/0.1% Triton X-100 for 1 h. TuJ1, a monoclonal antibody that recognizes the neuron-specific beta III tubulin isoform (gift of A. Frankfurter) was added at 1:1,000 at 4°C overnight, washed five times in PBS/0.01% Triton X-100, and then exposed to goat anti-mouse/biotin for 1 h at rt (Vector Labs, Inc., Burlingame, CA). The cells were washed five times in PBS/0.01% Triton X-100 and then the antibody was visualized using the ABC elite system with DAB and nickel from Vector Labs, Inc.

Results

Δ SCIP Peripheral Nerves Regenerate More Rapidly

We crushed the sciatic nerves of either adult wild type (wt) or Δ SCIP mice and then allowed the animals to recover for 1 wk. This time was selected because of the extensive degeneration distal to the crush point in wt animals that has been reported (Aguayo et al., 1973). We examined injured nerves at 5 mm below the crush site. In our hands, this distance from the crush site is largely free of new axons or new myelin 1 wk after injury. This is not unexpected even though established axons can grow at a rate of up to 1 mm/d (Hoffman and Lasek, 1975): the rate of growth cone extension is dependent upon numerous factors including intracellular calcium (Kater et al., 1988), growth factor concentration (Gundersen and Barrett, 1980) and extracellular matrix composition (Walter et al., 1990). In combination, these factors limit the overall rate of axonogenesis during regeneration since axon elongation is dependent upon growth cone extension.

Electron micrographs of wt nerve (Fig. 1 A) demonstrated many dead and dying axons, numerous profiles of Schwann cells autophagocytosing myelin (Fig. 1 A, *asterisks*), and an absence of regenerative profiles. The myelinated axons that were present had an appearance of being in the early stages of degeneration, as shown by their crenated morphology. In contrast, examination of the Δ SCIP mice at the same distance relative to the crush revealed an extensive degree of regeneration (Fig. 1 B), characterized by the appearance of thin, new myelin (Fig. 1 B, *arrowheads*), and corrugated basal lamina (bands of Büngner, herein termed “endotubes”) (Fig. 1 C). The corrugated, flaccid morphology of endotubes is due to the failure of the newly regenerated axons to attain a sufficient size to fill out the original volume of the endotubes. Over time, these axons and their associated myelinating Schwann cells will completely fill the endotubes, with an

associated loss of the corrugated appearance of the basal lamina (see below). The regenerating profiles, with the characteristic basal lamina seen in the Δ SCIP nerves present a markedly different appearance in contrast with the remaining myelinated axons in the wt mice, in which the basal lamina is still closely apposed to the myelin sheath. The unruffled appearance of the basal lamina in the wt mice is consistent with these being the original, myelinated fibers. Importantly, the axons from the Δ SCIP animals appear to be quite healthy, even at this early time point, with an apparent normal complement of cytoskeletal filaments and mitochondria (Fig. 1 C). We observed numerous profiles of regenerating structures and Schwann cells autophagocytosing their myelin (Fig. 1 B, *asterisks*). Profiles of both degenerating and regenerating fibers were present in the Δ SCIP animals, whereas only degenerating fibers were noted in the wt nerve. Table I demonstrates the difference in distributions of regenerating and degenerating axons in both the wt and Δ SCIP animals after crush injury. For a fiber to be considered a regenerating myelinated axon, the profile had to be ensheathed and wrapped by a thin myelin organelle and surrounded by a corrugated basal lamina. Such a structure is consistent with being an endotube remaining from a larger myelinated axon. It is noteworthy that the regenerating fibers are randomly dispersed amid profiles of degenerating axons, suggesting that the entire field has suffered extensive injury. As can be seen in the table, there is an absolute absence of regenerating myelinated fibers in the nerves of wt animals 1 wk after and 5 mm below the crush.

Both transgenic and wt animals were paralyzed ipsilateral to the crush. Partial paralysis can result from incomplete transection, but can appear to be a complete paralysis based on observation. To demonstrate that the paralysis observed in the operated mice was the result of complete mechanical axotomy and an associated loss of nerve function below the injury site, we have conducted electrophysiological testing of the animals 1 wk after injury ($n = 4$). These studies revealed an absence of nerve conduction in the sural nerves of the animals, further suggesting the completeness of the mechanical transection, and showing that regeneration had not yet advanced to the lower limb (Fig. 2). If the Δ SCIP mice had failed to degenerate in a timely manner, one might expect a retained physiological activity in the distal nerve in spite of the transection, which we failed to document. Contralateral to the injury, the animals had baseline conduction studies, consistent with exaggerated responses we have recently reported for the Δ SCIP mice (Bieri et al., 1997) (data not shown). Finally, we have compared very distal regions to verify that we are in fact documenting accelerated regeneration in the proximal nerve, as opposed to delayed degeneration, as is seen in the *ola* mouse (Glass et al., 1993; Glass and Griffin, 1994; Crawford et al., 1995). At sites \sim 10 mm below the injury, wt and Δ SCIP nerves are indistinguishable with respect to the degree of degeneration and the absence of regenerating profiles (Fig. 3). Taken en masse, the data show the crush injuries were complete, Wallerian degeneration had occurred as expected with an associated paralysis, and there was an established active axonal and myelin regeneration only in the Δ SCIP nerves at this early time point after crush. This is the first in vivo demonstration of a ge-

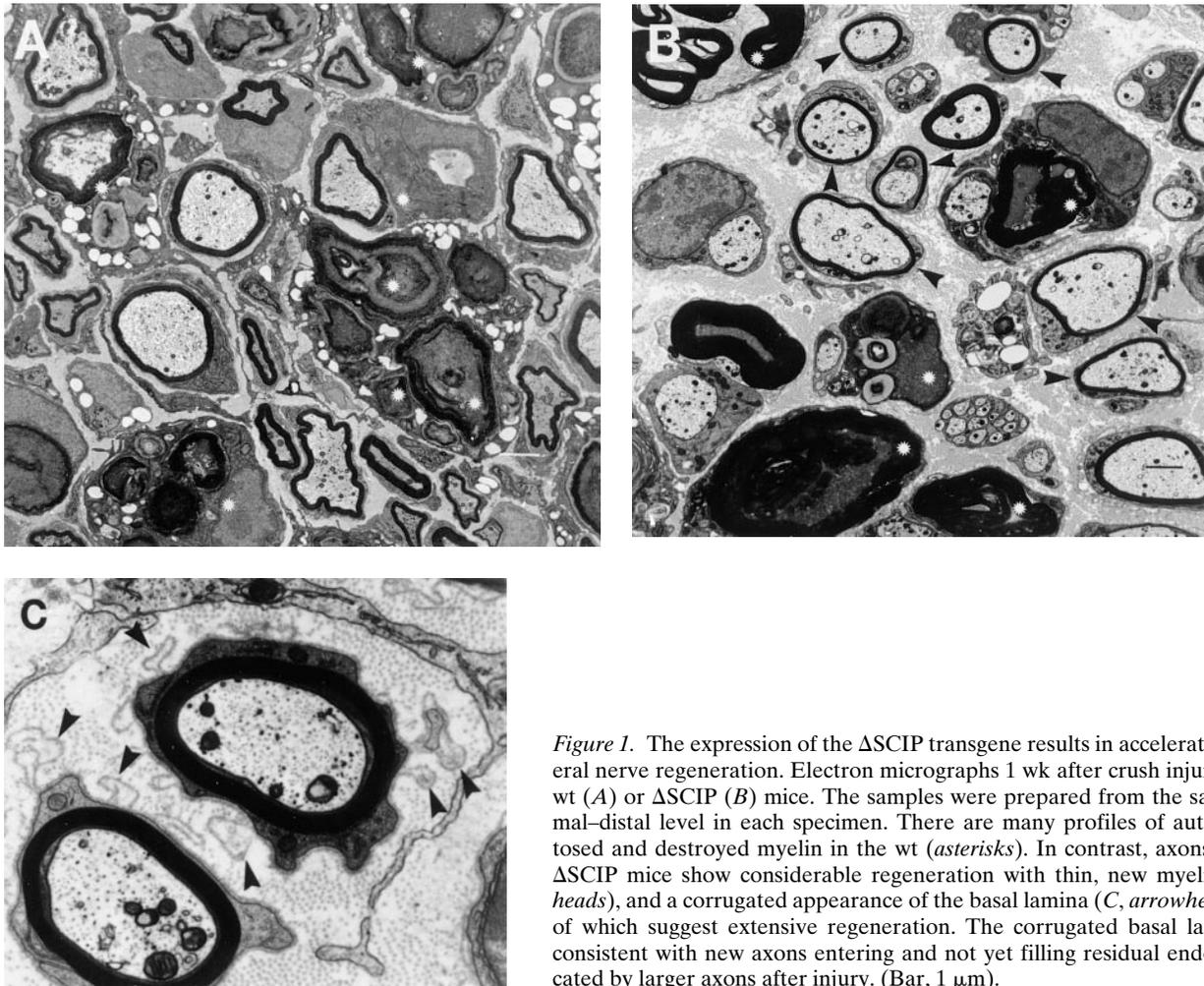


Figure 1. The expression of the Δ SCIP transgene results in accelerated peripheral nerve regeneration. Electron micrographs 1 wk after crush injury of adult wt (A) or Δ SCIP (B) mice. The samples were prepared from the same proximal–distal level in each specimen. There are many profiles of autophagocytosed and destroyed myelin in the wt (asterisks). In contrast, axons from the Δ SCIP mice show considerable regeneration with thin, new myelin (arrowheads), and a corrugated appearance of the basal lamina (C, arrowheads), both of which suggest extensive regeneration. The corrugated basal laminae are consistent with new axons entering and not yet filling residual endotubes vacated by larger axons after injury. (Bar, 1 μ m).

netically controlled acceleration of peripheral nerve regeneration.

The Δ SCIP transgene is expressed uniquely in the Schwann cells (Weinstein et al., 1995), and its effect on the accelerated axonal regeneration must therefore be indirect. This is consistent with previous observations that Schwann cells are both required for and are mediators of regeneration. Based on the above data it is clear that Schwann cells are capable of regulating the rate, as well as the extent of regeneration (see below). These data were

true of two lines of Δ SCIP transgenic animals, Δ SCIP line 1 and line 2. The data reported here are entirely from line 1, but have been demonstrated experimentally in both lines of Δ SCIP mice, thus ruling out the possibility that the described phenomenon is the result of an insertional event.

Axonal and Myelin Hypertrophy Mediated by the Δ SCIP Transgene

We next considered the long-term consequences of the expression of the Δ SCIP transgene on the regenerating peripheral nerve. To test this, we performed sciatic nerve crush surgery on wt and Δ SCIP animals, waited 30 d, and then harvested tissue for both biochemical assessment and electron microscopy. As expected, 1 mo after injury the wt nerve has largely regenerated (Fig. 4 A, WT). The axons have approached the size of the parent fibers (Fig. 4 B) and they were extensively myelinated. The thickness of this myelin is consistent with the previously described linear relationship between axonal diameter and myelin thickness (Friede and Samorajski, 1967). In contrast, the axons from the Δ SCIP animals have grossly surpassed their baseline dimensions (Fig. 4 A, Δ SCIP and B). The axons in these animals have hypertrophied such that their

Table I. Regenerating Myelinated Axons 1 Wk after Mechanical Transection

	Regenerating profiles (R)	Degenerating profiles (D)	R/D
Wild Type	0	458	0
Δ SCIP	357	284	1.257

Accelerated regeneration in the Δ SCIP animals 1 wk after crush. We have examined numerous fields of either wt or transgenic nerves 5 mm below the site of a mechanical transection. Here we present an enumeration of total regenerating and degenerating profiles, and calculate the ratio of new to dying fibers. To be considered a regenerating fiber, the axon had to be ensheathed and wrapped by a thin myelin organelle, and be surrounded by a corrugated basal lamina. In contrast, the profiles were deemed to be dead or dying by the presence of decaying or phagocytosed myelin, condensed axoplasmic contents, and/or a crenated appearance.

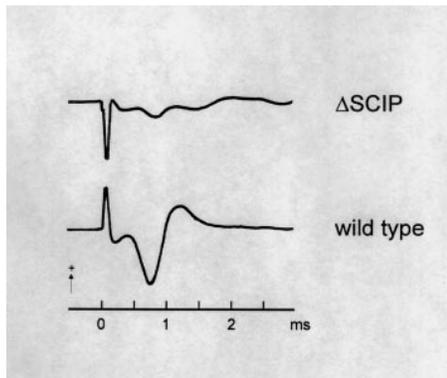


Figure 2. Loss of function correlates with an absence of electrophysiological function in the affected sural nerves after crush. 1 wk after unilateral sciatic nerve crush, all operated mice were paralyzed ipsilaterally to the nerve crush. Paralysis can result from incomplete transection; therefore, to determine the completeness of the mechanical transection we assayed for the presence of electrophysiological activity in sural nerves. There is an absence of electrophysiological response in either the Δ SCIP or wt sural nerve. If there was a delay in degeneration of the nerve distal to the crush injury or partial transection, there would be a measurable response in the sciatic nerve branches. However, no such activity is observed in either strain of mouse.

axonal diameters have not only overtaken the parent fibers, but have also grown well beyond the size of the wt axons (Weinstein et al., 1995) (Fig. 4 B, compare histograms). These data represent a radical change in the phenotype of the axons of the mutant animals in that they have progressed from significantly smaller than wt before injury, to much larger than wt axons after regeneration. Given the exaggerated size of the Δ SCIP axons after injury, it is not surprising that we found 21 times the level of neuron-specific tubulin in the Δ SCIP nerve preparations (Fig. 5, A and B). By definition, this axonal hypertrophy must be an indirect effect of the Δ SCIP Schwann cells and they alone express the transgene (Weinstein et al., 1995), suggesting an upregulation of Schwann cell-derived trophic support.

The myelin surrounding these fibers has also grossly hypertrophied, such that there was far more myelin per unit size axon than is expected (Fig. 4 A, compare panels) (Friede and Samorajski, 1967). This is consistent with, but an exaggeration of, the phenotype of the naive Δ SCIP animals in which the myelin is mildly hypertrophied with 150–200% more P₀ protein than in wt animals (Weinstein et al., 1995). Quantitative Western blotting of nerve-derived proteins 1 mo after injury revealed that the Δ SCIP sciatic nerves express 2,100% of the level of P₀ protein as compared to wt nerves at the same point in regeneration (Fig. 5, A and B). In addition, MBP expression is also dramatically elevated in the regenerating of Δ SCIP mice. Interestingly, not all Δ SCIP Schwann cell proteins have elevated expression patterns after regeneration. Connexin 32 is present at roughly the same level in both injured and contralateral nerves, and is the same in both wt and Δ SCIP animals (Fig. 5, A and B). This finding suggests that there are specific myelin-associated genes that are regulated by the SCIP pathway, whereas other myelin-associated genes lie

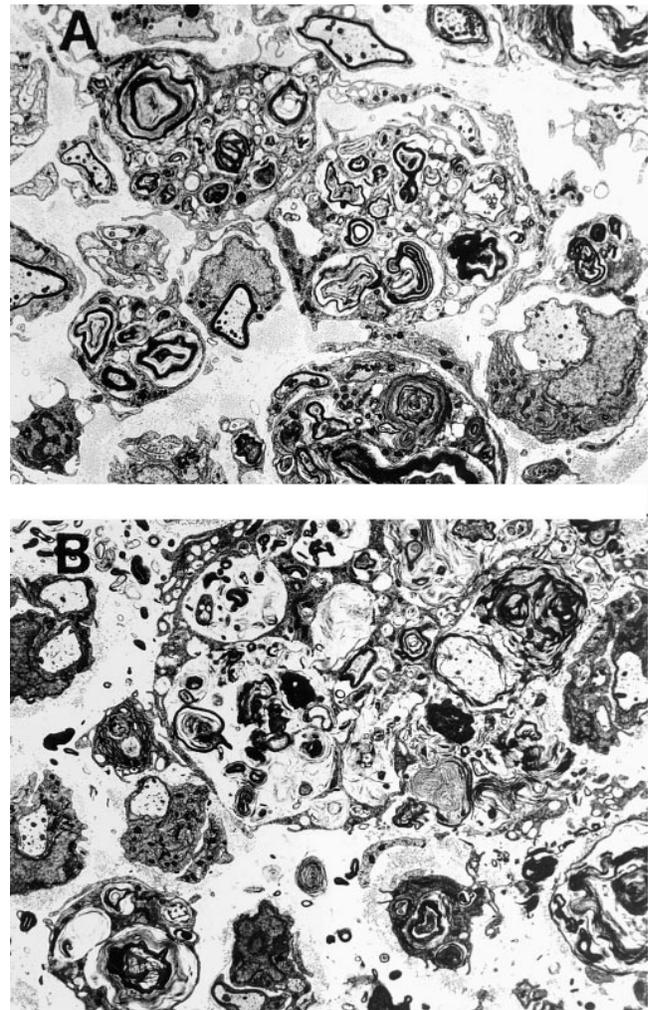


Figure 3. Equivalent degeneration in both Δ SCIP and wt mice 10 mm below sciatic nerve crush 1 wk after surgery. Regeneration of peripheral nerves follows a proximal to distal gradient. Therefore, the most distal nerve segments are the last to be reinnervated by new axons, and the last to undergo remyelination. The extent of degeneration in wt (A) and Δ SCIP (B) nerves is comparable at distances in which neither strain had effected regeneration. This observation rules out the possibility that there is a delayed degeneration in the animals that express this mutant form of SCIP, as well demonstrating the totality of the nerve crush. A delay in degeneration might account for the profiles observed in Fig. 1 if and only if there was an overall delay in degeneration. Such a delay would be accompanied by sustained electrophysiological function in the distal branches of the crushed nerve (refer to Fig. 2) and profiles on intact Schwann cell/axon units, neither of which is the case in these animals.

outside of activation in this pathway. The changes in morphology and upregulation of specific proteins do not appear to be the result in dramatic changes in expression of the Δ SCIP transgene itself, which was at equivalent levels ipsi- and contralateral to the lesion (data not shown).

Δ SCIP Schwann Cells Promote Axonal Outgrowth In Vitro

To directly test the possibility that the Δ SCIP Schwann

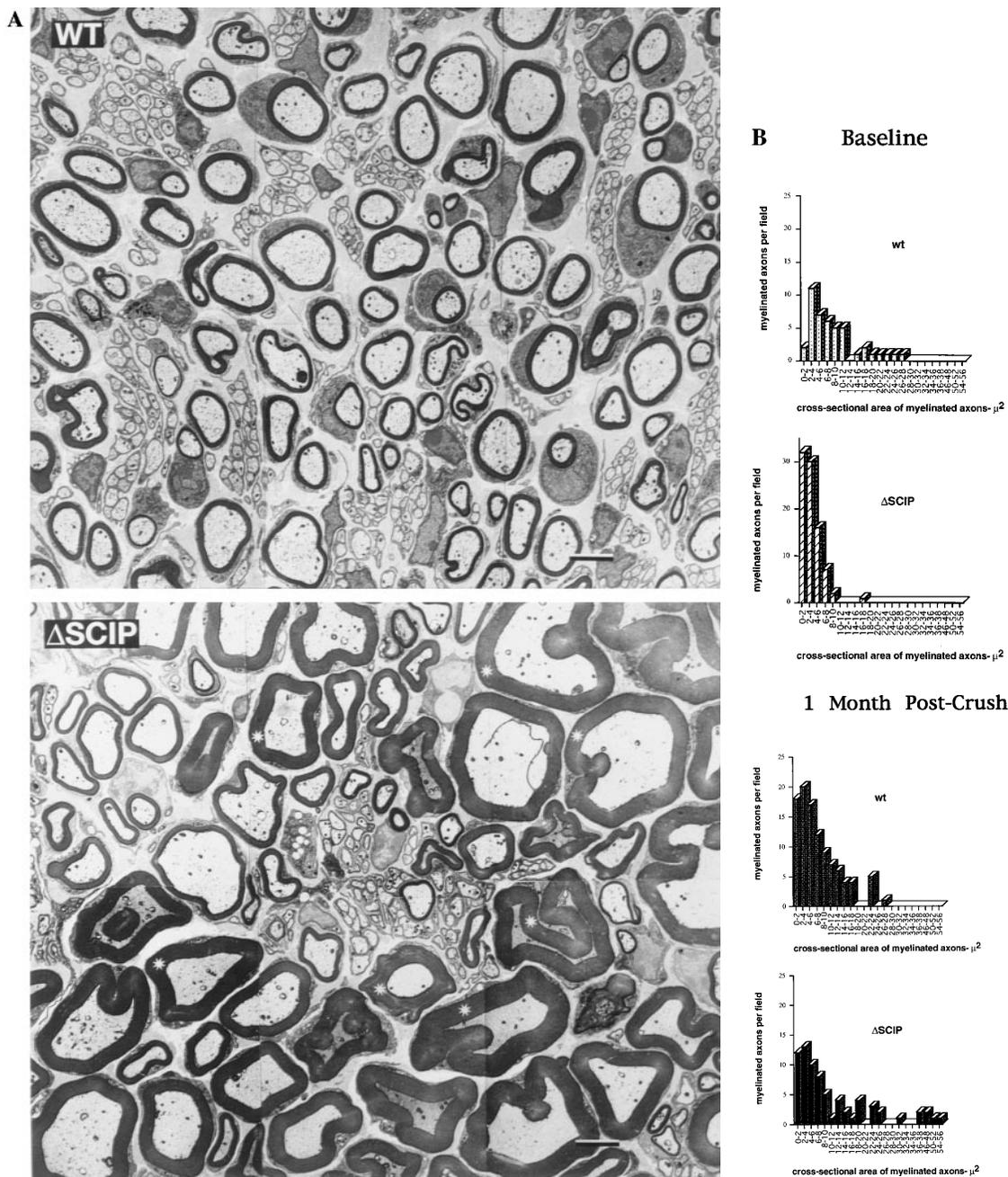


Figure 4. Axonal and myelin hypertrophy 1 mo after crush. (A) The montages are representative sections of sciatic nerve taken at the same axial level from either a wt or a ΔSCIP homozygote, and are displayed at the same magnification. The axons and associated myelin sheaths of the wt animals have approached the size of the original myelinated axons. However, in the ΔSCIP sciatic nerve the fibers are much larger than those in the wt animals. This is particularly dramatic because the ΔSCIP parent axons are smaller than wt fibers, thereby suggesting that the ΔSCIP Schwann cells are dysregulated and are oversupplying trophic support for their associated axons. In addition, the ΔSCIP Schwann cells have grossly overmyelinated their associated axons (*asterisks*), demonstrating a second dysregulation in these cells. (B) Axonal growth in the ΔSCIP mice after 1 mo of regeneration. A comparison of the axonal cross-sectional areas of myelinated fibers from regenerated nerves demonstrates the large size of the ΔSCIP axons after regeneration (from a $670\text{-}\mu\text{m}^2$ area). Several of the ΔSCIP myelinated axons exceed $30\ \mu\text{m}^2$, which is much larger than any of the wt myelinated fibers. The comparison of regenerated versus baseline myelinated axonal diameters demonstrates a phenotype switch in the ΔSCIP nerves. These axons transform from abnormally small fibers (ΔSCIP , baseline) to abnormally large axons (ΔSCIP , 1 mo after crush). Bar, $5\ \mu\text{m}$.

cells are the source of axon regeneration/promoting activity, we established cultures of either ΔSCIP Schwann cells or Schwann cells from wt littermates on postnatal d 2 as described (Brockes and Raff, 1979). Explants of neonatal

(P₀) rat DRG were harvested and cultured on top of the Schwann cell monolayers in the presence of saturating levels of NGF (100 ng/ml) in low serum-containing medium (1% FCS/0.5% horse serum). 20 h after coculturing, the

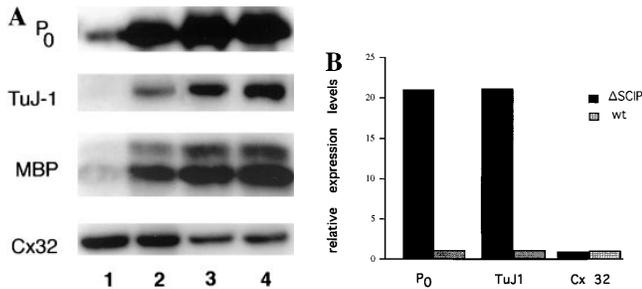


Figure 5. Overexpression of myelin and axonal proteins in the regenerating Δ SCIP nerve 1 mo after crush. (A) These are representative Western blots from proteins extracted from equal amounts of protein (15 μ g) from sciatic nerve comparing crushed (lanes 1 and 2) and contralateral, uncrushed (lanes 3 and 4) sciatic nerves from Δ SCIP (lanes 2 and 4) and wt (lanes 1 and 3). These data demonstrate a vast overexpression of P₀, neuron-specific tubulin and MBP in the regenerating Δ SCIP nerves 1 mo after injury. In comparison, there is roughly an equal amount of connexin 32 protein in all four samples. Sciatic nerve proteins were delipidated, resolved on a 12% SDS gel, and then transferred to nitrocellulose membranes. (B) A comparison of the density of the Western blotted bands for each of the proteins discussed in A. Samples are from the injured nerves only.

cells were fixed and stained with TuJ1, an antibody that recognizes neuron-specific tubulin (Easter et al., 1993), and therefore stains the DRG axons. Rat DRG neurons cultured on Δ SCIP Schwann cells have a greater number of axons, and these axons grow much farther than those from DRGs cultured on wt Schwann cells (Fig. 6, compare A and B). We have tested the Δ SCIP Schwann cells for overexpression of a number of molecules known to promote axonal survival and outgrowth. These include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), ciliary neurotrophic factor (CNTF), NGF, GGF2, and laminin, none of which is expressed above wt levels by the Δ SCIP cells (data not shown). Based on these data we believe that the Δ SCIP Schwann cells may be a convenient source of molecule(s) that have axon-promoting properties distinct from those currently described in the literature. The outgrowth-promoting activity is recoverable in the supernatant taken from cultured Δ SCIP Schwann cells. This activity is heat and trypsin sensitive, and is retained on 50-kD cut-off filters, all of which suggest that activity is dependent upon intact proteins (our unpublished observation).

Discussion

In these studies we have sought to understand the function of SCIP during peripheral nerve regeneration in a crush lesion model. SCIP is normally reexpressed by the Schwann cells after the regenerated axons contact them (Zorick, 1996). However, unlike the developing nerve, when SCIP is downregulated by the myelinating Schwann cells, its expression is maintained in the regenerated myelinating Schwann cells (Scherer et al., 1994). Our experiments were carried out in the Δ SCIP transgenic animals that express the POU domain of SCIP. The perturbation of SCIP function by the expression of the Δ SCIP in these

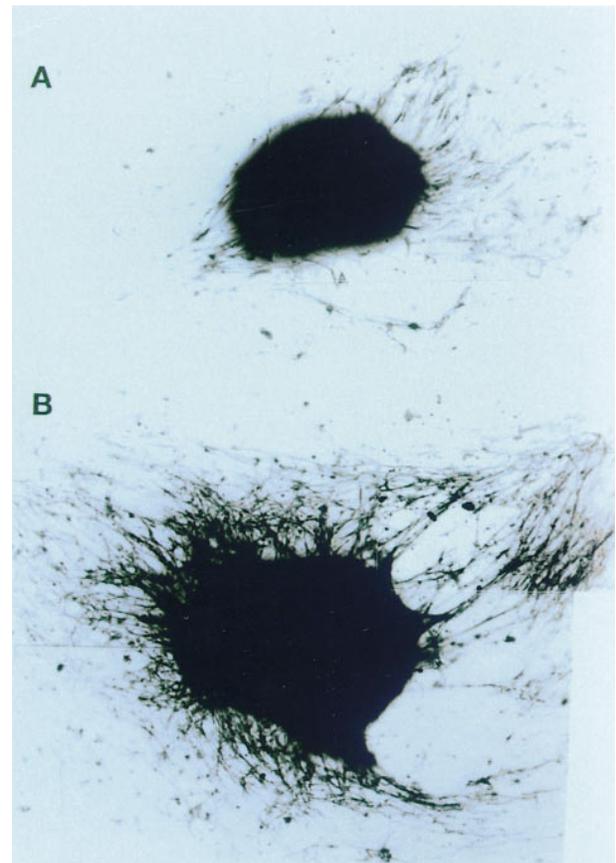


Figure 6. In vitro recapitulation of in vivo accelerated neuronal regeneration is mediated by Δ SCIP Schwann cells. Neonatal rat DRGs were plated on monolayers of either wt (A) or Δ SCIP (B) Schwann cells in saturating levels of NGF (100 ng/ml) and then grown for 20 h. The cultures were fixed and then the neurites and neuronal cell soma were stained with a neuron-specific anti tubulin-antibody, TuJ1. DRG neurons consistently extended more fibers that grew farther on Δ SCIP Schwann cells than on wt Schwann cells.

animals results in a dramatic increase in both the rate and extent of peripheral nerve regeneration. 1 wk after the experimental nerve crush, the axons from Δ SCIP transgenic animals have reentered at least 5 mm into the distal nerve and they have been remyelinated. In contrast, at the same time point and distance from the lesion, the wt animals were still actively undergoing degeneration without evidence of regeneration.

When the experiments were carried out for 1 mo after nerve injury, the Δ SCIP animals had vastly hypermyelinated the regenerated nerve, making >20 times the amount of P₀ protein as made by wt animals at the same stage of healing, which far exceeds the amount of myelin protein that the wt animals will ever make. The superinduction of the myelin structural genes in the regenerated Δ SCIP nerves is consistent in kind but not extent with our previous results. Those data demonstrated the myelin repressor function of SCIP was antagonized by the Δ SCIP transgene, which resulted in a twofold peripheral nerve hypermyelination by the Δ SCIP animals during development. However, the regenerated Δ SCIP nerve had 21

times the amount of P₀ protein when compared with the regenerated wt nerve. The exaggerated expression of the myelin proteins is consistent with the overelaboration of the regenerated myelin organelle in the Δ SCIP animals. Our observation that not all myelin genes are upregulated in this model demonstrates the specificity of the system, and suggests numerous regulatory controls in the complex interactions of Schwann cells and axons.

Taken together, the continuous expression of wt SCIP after nerve injury (Scherer et al., 1994) and the data presented in this report suggest that SCIP function is required for the establishment and maintenance of the regenerated myelin sheath. It also suggests that the Schwann cell in the regenerated nerve is intrinsically different from the original myelinating Schwann cell, in which SCIP is only transiently expressed and transiently required. Furthermore, these data suggest that the requirements for myelin homeostasis change between baseline, when SCIP is absent, and regeneration, when SCIP is expressed, and it implies that SCIP is limiting in the regenerated peripheral nerve. We have also documented indirect effects on the myelinated axons. The observed axonal hypertrophy represents a phenotypic switch between baseline (small axons) and regeneration (large axons). The very large axons appear to be healthy with a full array of filaments and mitochondria. These findings suggest a heightened level of Schwann cell-derived trophic support of the regenerated axons in the Δ SCIP mice, which is supported by data from *in vitro* neurite outgrowth studies, where the Δ SCIP Schwann cells were far superior to wt Schwann cells in promoting axonogenesis.

We have previously demonstrated that SCIP represses the P₀ and MBP genes, and that this repression is critical for the development of a normal peripheral nerve histoarchitecture and function (Weinstein et al., 1995; Bieri et al., 1997). However, in light of the data presented here it is difficult to posit a model in which the failure to repress myelin structural genes alone would result in the acceleration of regeneration, axonal hypertrophy, and the superinduction of myelin genes we observed. Alternatively, we believe that SCIP may be acting as a bifunctional molecule, depending on the state of the Schwann cell: a transcriptional repressor during development and a transactivator during regeneration. There is precedent for a positive transcriptional role for SCIP in the transactivation of the α 3 subunit of the acetylcholine receptor in neuronal cells via its POU domain (Fyodorov and Deneris, 1996). Notably, this is the domain of SCIP that is preserved in the Δ SCIP mutation. Diametrically opposed functions for SCIP in the same cell type have previously been postulated by Faus and colleagues in the keratinocyte, in which the activity of SCIP was inferred to differ depending on the state of the keratinocyte (Faus et al., 1994). We favor a hypothesis that in the regenerating Schwann cell, the Δ SCIP transgene, in consort with native SCIP, is behaving as a positive regulator of axon-promoting molecules, and possibly the myelin structural genes themselves. Whether the bifunctionality of SCIP results from a change in the expression pattern of interacting factors, a change in chromatin structure, or both is actively under investigation.

If the Δ SCIP Schwann cells offer superior support for the axon as demonstrated here, it raises the question of

why the axons in the naive Δ SCIP peripheral nerve are smaller than wt fibers. Based on the observation that there is extensive *in utero* myelination in the Δ SCIP pups, when both the fibers and the animals are still quite small (Weinstein et al., 1995), we reason that the developing axons are physically constrained and are unable to overcome the barrier that many turns of myelin pose. These immature, small axons would therefore be prevented from attaining their full potential diameter. Relief of that constriction, as demonstrated here by removal of the myelin sheath via a crush lesion, enables the axons to grow to their full size potential, which appears to be larger than wt fibers. Presumably the enlargement of the axons in the transgenic animals is under the control of Schwann cell-derived trophic support. Identification of the nature of this outgrowth-promoting activity will allow us to test these hypotheses more directly.

The data presented here are suggestive of possible therapeutic modalities in human disease. Schwann cells from sural nerve biopsies can be harvested, purified, and expanded in culture (Morrissey et al., 1995), and thus are susceptible to genetic manipulation *in vitro*. The human homologue of SCIP has been cloned and it is >90% identical to both rat and mouse SCIP (Tobler et al., 1993). The introduction of a human variant of the Δ SCIP transgene can be accomplished by either transfection or retroviral infection, and the altered Schwann cells can be used in an autologous transplantation paradigm. This type of approach might be useful in peripheral nerve regeneration as well as spinal cord trauma. Schwann cells invade the spinal cord and myelinate central axons (Blight and Young, 1989) as well as permit restored function (Gilmore and Duncan, 1968; Snyder et al., 1975) after spinal cord injury, making this an attractive model system to test the axon-promoting activity of the Δ SCIP Schwann cells.

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