Journal of Cellular Biochemistry 88:970–985 (2003)

Identification of Reciprocally Regulated Gene Modules in Regenerating Dorsal Root Ganglion Neurons and Activated Peripheral or Central Nervous System Glia

Adrian A. Cameron, Gordon Vansant, Wen Wu, Dennis J. Carlo, and Charles R. ILL*

The Immune Response Corporation, 5935 Darwin Court, Carlsbad, California 92008

Abstract Differential gene expression in the rat after injury of dorsal root ganglion neurons in vivo, and simulation injury of Schwann cells and oligodendrocytes in vitro was analyzed using high-density cDNA microarrays. The analyses were carried out to study the genetic basis of peripheral nerve regeneration, and to compare gene regulation in glia of the central (oligodendrocyte) and peripheral (Schwann cell) nervous systems. The genes showing significant differential regulation in the three study groups represented all aspects of cellular metabolism. However, two unexpected observations were made. Firstly, a number of identical genes were differentially regulated in activated Schwann cells, activated oligodendrocytes and regenerating DRG neurons. Specifically, a group of 113 out of 210 genes that were down-regulated in Schwann cells upon lipopolysaccharide (LPS) treatment, were identical to genes up-regulated in the injured, regenerating DRG. Furthermore, a group of 53 out of 71 genes that were down-regulated in interferon gamma (IFN-γ)/LPS-activated oligodendrocytes, were identical to genes up-regulated in the DRG neurons. Finally, 22 genes were common to these three groups, i.e., down-regulated in activated oligodendrocytes, down-regulated in activated Schwann cells, and up-regulated in regenerating DRG neurons. Secondly, a group of 16 cell-cycle and proliferation-related genes were up-regulated in the DRG following sciatic nerve crush, despite the absence of cells undergoing mitosis in the DRG, or any significant presence of apoptosis-related gene expression. Therefore, it appears that in these three cell types, large sets of genes are reciprocally regulated upon injury and/or activation. This suggests that the activation of the injury-related gene expression program in cell derivatives of the neuroectoderm involves, in part, highly conserved genetic elements. J. Cell. Biochem. 88: 970–985, 2003.

Key words: sciatic; transcription; mrna; regeneration; gene; lipopolysaccharide; spinal cord

Early studies on regeneration of the mammalian peripheral nervous system revealed that following a crush injury, the axons of the peripheral nerve were able to regenerate their axons into the distal segment of the nerve [Cajal, 1928]. Subsequent studies showed that this capacity was based on two phenomena. The first is an anabolic response within the neurons of the dorsal root ganglia (DRG) [Perry and Wilson, 1981], that is dependent on gene transcription [Wells and Vaidya, 1994]. Specifically, this includes expression of transcription factors [Leah et al., 1991] and growth-related genes like GAP-43 and galanin [Vilar et al., 1989; Groves et al., 1996]. The second component is an inflammatory response that occurs within the distal segment of the injured nerve. The key components of this inflammatory response include invasion of the nerve by macrophages that engulf the myelin sheath and damaged axonal processes [Hirata et al., 1999], and proliferation of Schwann cells that surround the nerve. The proliferating Schwann cells secrete neurotrophic factors, cell adhesion molecules and extracellular matrix proteins [Fu and Gordon, 1997]. Together, the anabolic response in the DRG and the inflammatory response at the injury site result in successful regeneration.

By contrast, a crush injury to the central nerves in the spinal cord is not followed by regeneration [Cajal, 1928]. Although the upper motor neurons of the corticospinal tract mount a transcriptional response [Tseng and Prince, 1996], and can survive long-term axotomy

*Correspondence to: Charles R. ILL, Cancervax Corporation 2110 Rutherford Road Carlsbad, CA 92008.
E-mail: rill@cancervax.com

Received 6 September 2002; Accepted 16 September 2002
DOI 10.1002/jcb.10392

[Giehl and Tetzlaff, 1996], the beneficial inflammatory response of the peripheral nerve is essentially absent. The mature myelinating cell of the CNS, the oligodendrocyte, is susceptible to apoptosis following trauma [Shuman et al., 1997]. Furthermore, specific components of the myelin sheath produced by oligodendrocytes have inhibitory effects on axonal elongation [Caroni and Schwab, 1988; Chen et al., 2000]. The complimentary, synchronized responses of DRG neurons and Schwann cells following a crush injury suggest that the process of peripheral nerve regeneration is tightly coupled. The failure of regeneration following axotomy of central neurons indicates that this same highly orchestrated chain of events in the periphery is lost in the mammalian CNS, possibly as a result of the sequestration of the beneficial (Th2) inflammatory component from the CNS compartment [Moalem et al., 2000]. In order to gain some understanding of the specific factors influencing this profound difference, we sought to compare the genetic profiles of regenerating DRG neurons and activated Schwann cells from the PNS, as well as activated oligodendrocytes from the CNS.

The study of differential gene expression has been greatly advanced by the development of high-density microarrays [Schena et al., 1995]. In conjunction with the identification and sequencing of the >30,000 genes of the human genome [Lander, 2001; Venter, 2001], the development of microarrays has enabled the measurement of gene expression responses to a variety of stimuli including disease, trauma, and environmental toxins. As a result, it is possible to identify clusters of genes of related function or expression level, as well as the patterns of gene expression which occur as a function of time [Wen et al., 1997]. We have applied this technology to a number of models of peripheral and central nervous system trauma [Farlow et al., 2000], in order to compare the patterns of gene expression in activated central and peripheral neurons and glial cells.

The current study sought to compare differential gene expression in axotomized regenerating DRG neurons, LPS treated Schwann cells, and LPS/IFN-γ-treated oligodendrocytes. Since in the DRG, the neurons are likely to be one of the most transcriptionally active cells following distal peripheral nerve injury, mRNA was extracted from whole DRG 7 days after a crush injury to the sciatic nerve in vivo. Because fibroblasts [Salonen et al., 1988], macrophages [Toews et al., 1998] and vascular cells [Podhajsky and Myers, 1993] are all present at or distal to the peripheral injury, sciatic nerves were extracted from rat pups, and their associated Schwann cells were cultured to purity. Schwann cells were exposed in vitro to bacterial LPS, to approximate their activated state in a degenerating nerve segment. Oligodendrocytes from the spinal cord of neonatal rat pups were cultured to purity, and activated by exposure to LPS and IFN-γ. The mRNA from these three tissues, together with the respective controls was purified and converted to cDNA, which was hybridized to three cDNA microarrays containing sequences of ~10,000 human genes. The expression levels of 545 genes changed significantly in the DRG, 450 genes were changed in the treated Schwann cells, and 78 genes were changed in activated oligodendrocytes. In the DRG, 95% of the genes were upregulated and 5% were down regulated. In the Schwann cells, 54% were up-regulated and 46% were down-regulated. In oligodendrocytes, 91% were down-regulated and 9% were up-regulated. These models have been published in preliminary form [Farlow et al., 2000] but the actual data are analyzed and expanded upon herein and shown in detail at (http://www.imnr.com/microarray.htm).

METHODS

Surgical Procedures

Experiments were performed on 36 Sprague–Dawley rats, female, 200–250 g. The animals were anesthetized with a mixture containing 22% ketamine, 13% xylazine, and 2.5% acepromazine in saline (1.6 ml/kg, i.m.). In 15 animals, the sciatic nerves were exposed bilaterally in the mid-thigh region, and crushed twice for 20 s with No. 5 jeweler's forceps [Jacob and McQuarrie, 1996]. Confirmation of the disruption of the nerve fibers was made by a change in coloration/indentation at the crush site, and an absence of twitching response to nerve pinch peripheral to the crush site several minutes post-injury. The lesion site was cleaned by lavage with sterile saline and the skin closed with Michel clips. In another 15 animals, the sciatic nerves were exposed bilaterally, the site cleaned by lavage with sterile saline, and the skin was closed with Michel clips (sham operation). The animals from each experimental group (15 nerve-crushed and

15 sham-operated) were initially operated on in groups of five on 3 consecutive days. Thus, 7 days after the initial operation, a group of five animals was sacrificed and the L4 and L5 dorsal root ganglia were quickly exposed, removed, rinsed briefly in phosphate buffered saline (PBS) and frozen in liquid nitrogen, prior to storage at −70°C.

Bromo-Deoxyuridine (BrdU) Immunohistochemistry

Six additional rats (3 × 7-day post nerve-crushed and 3 × 7-day post sham-operated) were used for immunohistochemical studies of BrdU uptake. The animals were injected with BrdU (Sigma, St. Louis, MO 50 mg/kg) 2 h prior to re-induction of anesthesia and perfusion of chilled PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The intestines, and L4 and L5 DRG were dissected out and fixed for 4 h, followed by cryoprotection in 30% sucrose in 0.1 M PB. The DRG and intestines were sectioned in a cryostat at 10–15 μm, and collected in 0.1 M PB. For immunohistochemistry, the sections were processed in accordance with the protocol supplied by the manufacturer (product no. 1170376, (http://biochem.roche.-com/pack-insert/1170376A.pdf).

Schwann Cell Culture

Sprague–Dawley rat pups (10 × 3-day-old pups) were anesthetized by cryo-anesthesia (immersion in crushed ice), and the sciatic nerves were dissected out bilaterally. The pups were then sacrificed. Primary cultures of Schwann cells were established as described previously [Brockes et al., 1979, Montgomery et al., 1996]. Pure, confluent cultures were activated with bacterial lipopolysaccharide [Skundric et al., 1997].

Oligodendrocyte Culture

Sprague–Dawley rat pups (10 × 3-day-old pups) were anesthetized by cryo-anesthesia, and the spinal cord was carefully dissected out. The pups were then sacrificed. Oligodendrocyte cultures were established using methods modified from Van der Pal et al. [1990]. In contrast to Schwann cells, oligodendrocytes require additional stimuli to effect activation. Therefore, cultures of differentiated oligodendrocytes were activated with LPS and IFN-γ as described [Hewett et al., 1999].

mRNA Purification and Microarray Hybridization

The ganglia (60 DRG from 15 nerve crushed animals, and 60 DRG from 15 sham operated animals) were homogenized and the total RNA was extracted using Trizol, following the procedure supplied by the manufacturer (Invitrogen, Carlsbad). From this, the mRNA fraction (an average of 10 ng mRNA was extracted from each DRG) was purified using an oligo-dT column, following the procedure supplied by the manufacturer (Qiagen, San Diego, CA). For Schwann cells, and oligodendrocytes the LPS-activated and LPS/IFN-γ cultures, respectively, were rinsed with PBS, and the RNA extracted and stabilized by rinsing the flasks with 3 ml Trizol, which was then stored at −70°C. The mRNA fraction was purified using an oligo-dT column following the procedure supplied by the manufacturer (Qiagen).

Following mRNA purification, the experimental and control fractions of DRG, oligodendrocytes and Schwann cells, the purified mRNA was supplied to Incyte, Inc., for the reverse transcription to cDNA and fluorescent labeling using Cy5 and Cy3-labeled random primers, respectively. The reactions were incubated for 2 h at 37°C with 200 ng poly-A RNA, 200 U M-MLV reverse transcriptase (Invitrogen, Carlsbad), 4 mM DTT, 1 U RNase inhibitor (Ambion), 0.5 mM dNTPs, and 2 μg Cy3 or Cy5-labeled 9-mers in 25 μl volume with enzyme buffer supplied by the manufacturer. The reaction was terminated by incubation at 85°C for 5 min. The paired reactions were combined and purified with a TE-30 column (Clonetech, Palo Alto, CA). They were then simultaneously hybridized to a microarray containing multiple copies of each of 10,000 double-stranded human cDNA fragments with an average length of 1,000 base pairs (bp) (Unigem-1; Incyte Corp., Palo Alto), corresponding to known genes (6,600) and unknown sequences (ESTs) (3400) held in GenBank. Arrays containing human sequences were used because "rat arrays" had not been developed at the time the experiments were performed. The control and experimental fractions were hybridized to the array with 5 × SSC at 60°C for 6.5 h. The arrays were washed with 1 × SSC and 0.1% SDS at 45°C for 10 min, and then with 0.1 × SSC and 0.2% SDS at 20°C for 3 min.

For each gene on the microarray, quantitative differences between the hybridization of

experimental and control cDNA species (reverse transcribed from the mRNA populations and labeled with Cy3 or Cy5 dyes) were measured by using confocal microscopy and digital imaging with 10 μm resolution. Both Cy3 and Cy5 channels were simultaneously scanned with independent lasers. The fluorescent light was optically filtered before multiplication, and conversion to a 16 bit digital signal. A 16-log scale was used for visual representation.

The level of differential expression for each gene/array element was calculated in two steps. Firstly, a balance coefficient was calculated, which was the ratio of the average Cy3 signal (across all elements) to the average Cy5 signal (across all elements). The Cy5 (experimental) channel of each element was then multiplied by the balance coefficient. Secondly, the differential expression ratio was calculated for those elements having a signal-to-noise ratio of at least 2.5. For elements with Cy3/Cy5 ratio of >1 (control > experimental) the differential expression = Cy3/Cy5. For elements with Cy3/Cy5 ratio <1 (control < experimental), differential expression $= -$(Cy5/Cy3). All genes with a balanced differential expression ratio of $> +1.9$ or < -1.9 were included for analysis. Each array contained internal controls for quality assurance of the following: accuracy of RNA ratio (-3 to $+25$), sensitivity of detection of RNA (from 2 pg to 2 ng), determination of hybridization efficiency, sample RNA quality (cDNA targets from a variety of rat tissues) and house-keeping gene constancy (human alpha tubulin, human ribosomal protein p9, and 23 kDa HBP).

The data generated consisted of the change in expression level, and IMAGE. Consortium numbers corresponding to the cDNA sequences of the microarray to which reverse transcribed mRNA sequences from the samples had bound. From the IMAGE Consortium numbers, the GenBank Accession numbers were obtained, and homologies to known genes were then determined through the use of the BLAST bioinformational database.

Validation of Microarray Result

In order to test the reproducibility of the differential expression, and the impact of sample variability on the precision of differential expression ratios, the manufacturer (Incyte Genomics, Inc.) performed 10 hybridizations of duplicate samples of each of three tissue types (heart, brain, and placenta) [Yue et al., 2001]. In three additional control experiments, the coefficient of variation of the differential expression ratios was calculated on the basis of (a) reverse, fluorescent labeling of the identical mRNA samples, (b) the visual rendering of the signal (a measure of the performance of signal detection equipment and measurement equipment), and (c) genes which did not have expression ratios of 1:1 (i.e., 10 hybridizations of each of brain vs. placenta, heart vs. brain, heart vs. placenta). In all four experiments, the coefficient of variation of differential expression was approximately 12% [Yue et al., 2001]. The accuracy of Incyte Unigem arrays has recently been verified by Mirnics et al. [2000] who showed, following hybridization of the same cortical mRNA sample, that at the 99% confidence level (differential expression of $> +1.9$ or < -1.9), 4 out of 4,844 genes were false positives, and 2 out of 4,484 were false negatives. The same authors hybridized a control and experimental sample of cortical mRNA to 3 Incyte Unigem arrays and obtained the same differential gene expression data in each case [Mirnics et al., 2000]. These data confirm the accuracy and reproducibility of data obtained using the Incyte Unigem arrays. We performed semi-quantitative PCR analysis designed to validate the array as described below.

RT-PCR Analysis of Selected Differentially Displayed Genes

Approximately 205–225 ng of the same mRNA used for the array hybridization was used as template for the RT (reverse transcription) reaction that was carried out as follows. Any DNA present was removed from the mRNA samples by exposure to DNase at 37°C for 20 min followed by the addition of 1/10 volume of 25 mM EDTA, and a temperature increase to 60°C for 10 min. Each reaction was divided into two and annealed to an oligo-dT at a temperature of 70°C for 10 min, followed by cooling to 4°C. cDNA was produced by adding Superscript II reverse transcriptase (Invitrogen, Carlsbad) to one half of each of the mRNA samples in a solution containing 15 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, and 10 mM DTT. Transcription was carried out for 50 min at 42°C. The other half of the mRNA samples contained everything except the transcriptase as a control for DNA contamination. Two microliters of each reverse transcription (RT) reaction was used as template for the polymerase chain

reaction (PCR). Each PCR reaction contained primer pairs that would amplify a selected gene found on the microarray. Each reaction was carried out in 15 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, and 2.5 U of amplitaq gold (Applied Biosystems) using the following programs; 10 min at 94°C (to activate the amplitaq gold), linked to a program that had a cycle of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min and 30 s. The second program was allowed to cycle 30 times before lowering the temperature to 4°C. The primers pairs for each reaction are as follows; EST (Acc. no. H52429) sense: GTCTCGCTATGTTGCCCA, EST (Acc. no. H52429) antisense: GCCTGTAG-TCCCAGCTAC; glycogen synthase kinase (Acc. no. H64697) sense: CGCCAGA CACTATAGTC-G; glycogen synthase kinase (Acc. no. H64697) antisense: CTCTGGTGCCCTGTAGTA; beta-actin sense: TCACCCACACTGTGCCC ATCTA-CGA; beta-actin antisense: CAGCGGAACCGC TCATTGCCAATGG. Twenty percent of each reaction was run out on a 1.5% agarose gel. The signal was standardized against the house-keeping gene beta actin. The gel images were quantified using the Metamorph digital imaging program.

RESULTS

Microarray Validation

While validation of the microarray has been extensively performed by the manufacturer [Yue et al., 2001] and by other groups [Mirnics et al., 2000, see Materials and Methods], we performed an independent test designed to validate the accuracy of the array. As shown in Figure 1, a reverse transcriptase reaction was performed on the same mRNA sample as that used for the hybridization. Using this cDNA, PCR was performed using primers derived from the sequence of glycogen synthase kinase that was predicted by the array to be 3.4-fold lower on the nerve-crushed side. This revealed a product of approximately 300 bp, that upon image-analysis quantification, was × 3 lower in the experimental sample than in the control sample (Fig. 1A, lanes 5 and 3, respectively). When PCR was performed on the same template using primers derived from the EST no. H52427

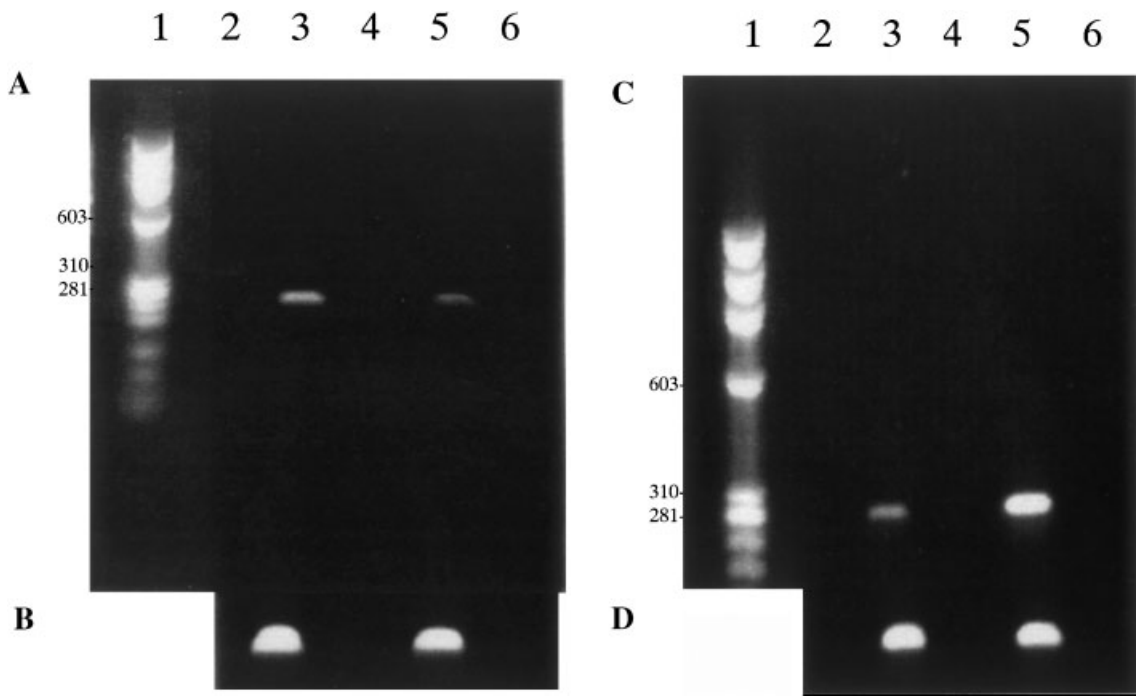

Fig. 1. Validation of array data by RT-PCR. **A**: Ethidium bromide staining of a gel containing a PCR product formed from glycogen synthase kinase (Acc. no. H64697), which was predicted by the microarray to be down-regulated 3.4-fold. Nerve crush and control samples are depicted in lanes 5 and 3, respectively. **B**: Actin control for normalization of loading of samples. **C**: Ethidium bromide staining of a gel containing a PCR product formed from EST (Acc. no. H52429) predicted by the array to be up-regulated 39-fold. Nerve crush and control samples are depicted in lanes 5 and 3, respectively. **D**: Scan of the actin control, showing loading of samples.

predicted by the array to be up-regulated 39-fold on the nerve-crushed side, a product of 295 bp was produced. Upon image-analyzer quantification, this product was $\times 35$ higher in the experimental sample than the control sample (Fig. 1C, lanes 5 and 3, respectively). The values were normalized to the actin controls by digital imaging analysis (Fig. 1B,D). These results confirm the predictions made by the microarray (EST Acc. no. H52427, up-regulated), glycogen synthase kinase (Acc. no. H64697, down-regulated), and validate its accuracy.

Characteristics of Gene Changes in Regenerating DRG Neurons, Activated Schwann Cells, and Activated Oligodendrocytes

The proportion of genes significantly modulated in DRG neurons, Schwann cells, and oligodendrocytes is shown in Table I. Thus for DRG neurons, 521 genes (95%) were significantly up-regulated and 25 (5%) were significantly down-regulated. The expression range was $+39$ to -4. In the activated Schwann cells 242 genes (54%) were up-regulated and 210 (46%) were down-regulated. The dynamic range was $+7$ to -7. In activated oligodendrocytes, 8 genes (10%) were up-regulated and 71 genes (90%) were down-regulated. The expression range was $+7$ to -4. Thus it appears that there are three main features of the distribution of differentially regulated genes. Firstly, there were many more genes modulated in DRG neurons (545 genes, peripheral origin) and Schwann cells (452 genes, peripheral origin) that in oligodendrocytes (78 genes, central origin). Secondly, there are significant differences between the expression range of genes in DRG neurons ($+39$ to -4) compared with either of glial cell types ($+7$ to -7 in Schwann cells, $+7$ to -4 in oligodendrocytes). Finally, there are marked differences among all three cell types concerning the distribution of up and down-regulated genes, with the DRG neurons and oligodendrocytes having reciprocal distributions (95% up- and 5% down-regulated in DRG, 9% up- and 91% down-regulated in oligodendrocytes), while Schwann cells were more evenly distributed. This is shown graphically in File 1 on the website http://www.imnr.com/microarray/histogram.pdf created for the inclusion of all associated microarray data.

The identities of all the genes with expression level changes of greater than $+1.9$-fold and less than -1.9 were determined using the BLAST bioinformatic database. It was observed that these genes reflected activity in a large variety of cell functions, including nuclear, membrane, intermediary metabolism, secreted proteins, signaling/regulatory, organelle, cell cycle, redox metabolism, and apoptosis. The percentage of genes in each of the various categories for each of the three cell types is shown in Tables A–C (File 2) on the website located at: (http://www.imnr.com/microarray/celldistribution.pdf). In DRG neurons, Schwann cells, and oligloden-drocytes, 65, 61, and 61% respectively, of all significantly changed genes were comprised of nuclear, membrane, metabolic and secreted proteins. All known genes from these three cell types that encoded membrane, secreted or nuclear proteins, together with their differential expression levels are shown in File 3 of the website located at: (http://www.imnr.com/microarray/genelist.pdf).

Within the DRG group of modulated genes two subgroups were identified because of their relevance to the regeneration process. Firstly, we identified the modulated genes encoding proteins in signal transduction pathways. The pathways include those associated with members of the Ras superfamily of GTP-binding proteins (Fig. 2A) and those associated with the MAP kinase pathway (Fig. 2B) both of which are discussed below. Secondly, it was found that a total of 16 genes were up-regulated that have previously been identified with membranes of cancer cells (proliferating cells), or nuclear

TABLE I. Number of Genes Significantly Modulated (Up- or Down-Regulated by a Factor of 2 or More) in Regenerating DRG Neurons, Activated Schwann Cells or Activated Oligodendrocytes

	DRG	Schwann cells	Oligodendrocytes
Up-regulated	521 (95%)	242 (54%)	8 (10%)
Down-regulated	25 (5%)	210 (46%)	71 (90%)
Expression range	+39 to −4	+7 to −7	+7 to −4

A

Gene Modulated	Gtp-Coupled Protein	Effector Function	Location
IQGAP2 (x5) (Acc. No. T86898)	Cdc42-GTP	Cytoskeleton	Cytoplasm
Radixin (x2) (Acc. No.H80175)	Rho-GTP	Cytoskeleton	Cytoplasm
ARL-3 (x12) (Acc. No.AA011142)	ARF-GTP	Unknown	Cytoplasm
Ran-BP2 (x3) (Acc. No. AA001246)	RAN-GTP	Nuc.-Cyt. T'port	Nucleus
Karyopherin B3 (x3) (Acc. No. N78664)	RAN-GTP	Nuc.-Cyt. T'port	Nucleus

B

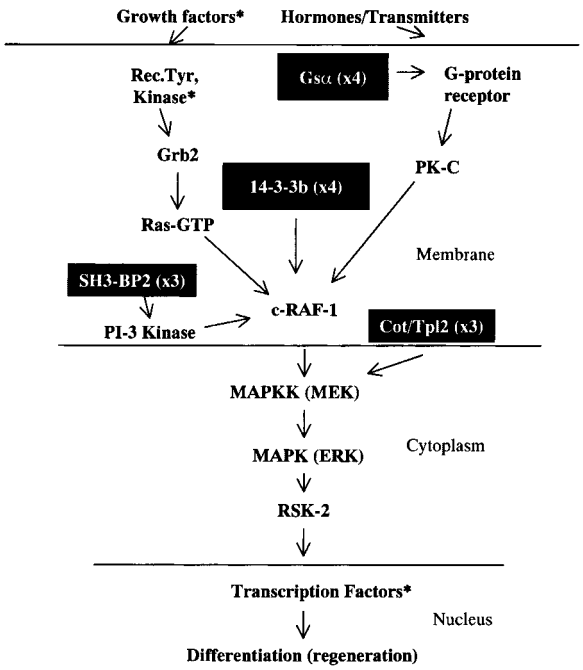

Fig. 2. Identification of genes encoding intracellular signaling proteins within the regenerating DRG. **A**: Genes that modulate GTP-coupled proteins during nerve regeneration. **B**: Genes predicted by the array to influence elements of the MAP-kinase pathway. In the extracellular space, growth factors and neurotransmitters activate receptor tyrosine kinases and G protein-coupled receptors, respectively. The receptors activate the intermediates Ras-GTP or protein kinase C, which can activate c-Raf (MAP-KKK). In addition, phosphoinositol-3-kinase (PI-3) can be recruited to the activated receptor tyrosine kinases. Genes up-regulated in the array SH3-BP2 and 14-3-3 modulate activity of PI-3 kinase and C-raf, respectively. Tpl-2 a novel MAP-KKK can regulate the activity of MAP-KK (MEK). This protein activates in turn MAPK (ERK) and RSK-2, which translocate to the nucleus and activates transcription factors (listed on website: http://www.imnr.com/microarray/genelist.pdf), which control the production of mRNA necessary for peripheral nerve regeneration. Expression level changes for each gene shown in parentheses. MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase.

TABLE II. Genes Encoding Proliferation Antigens and Cell Cycle Regulators in L4/5 Dorsal Root Ganglia Following Sciatic Nerve Crush

Cancer cell antigens		
Calgizzarin	(R76776)	(12.6)
Melan-A	(N32199)	(5.0)
MUC 18	(M28882)	(5.0)
EVI-5 hom.	(H29381)	(4.6)
TI-227	(AA053771)	(3.7)
MAGE-8	(AA045931)	(3.5)
MAC-30	(N33221)	(3.1)
SAS	(W96199)	(3.0)
Cell cycle transition		
EB1	(AA035277)	(4.6)
Mutated p53	(H61357)	(4.5)
Host cell factor-1	(W93018)	(4.1)
Geminin	(N55327)	(3.8)
HERV-K	(N95674)	(2.6)
Cyclin F	(T89627)	(2.2)
Cdc 34	(AA045671)	(2.3)
Cdk-kinase	(AA031961)	(−2.8)
Cell-cycle genes not significantly changed on microarray		
Cyclin isoforms: A2, B1, B2, D1, D2, E1, G2, H, K, I		
Cyclin-dependent kinases: 2, 4, 5, 6, 8, 9, 10		
Cyclin-dependent kinase inhibitors: 1C, 2A, 2C, 2D, 3		

Numbers in parentheses indicate accession number and magnitude of change. Also shown are cell-cycle genes included on the array that were not significantly up- or down-regulated.

proteins which are involved with regulation of the cell cycle (Table II). Despite the up-regulation of a number of proliferation-related genes, other genes which are required for mitosis and which were present on the array, were not significantly changed (Table II). The presence, however of these proliferation-associated genes raised the possibility that mitosis could be occurring in some cells within the DRG. To test for mitosis in the DRG, cryostat sections of DRG and intestine (for controls) from seven-day post nerve crush and sham-operated animals were immunostained for the nuclear mitotic marker bromodeoxy-uridine (BrdU) (Fig. 3). In sections of intestine, BrdU stained cells can be seen in the crypts of the intestinal villi (Fig. 3A). The staining is absent in control sections of intestine (Fig. 4B). In sections of DRG from a nerve-crushed animal (same animal as in Fig. 3A,B) no evidence of BrdU staining was observed (Fig. 3C). Similarly, in sections of a sham-operated animal, BrdU-stained cells were not observed (Fig. 3D). These results indicate that cell division was absent in the DRG 7 days after sciatic nerve crush, and in turn suggests that the expression of these proliferation-associated genes may be linked to some other function in the genetic program of the regenerating DRG.

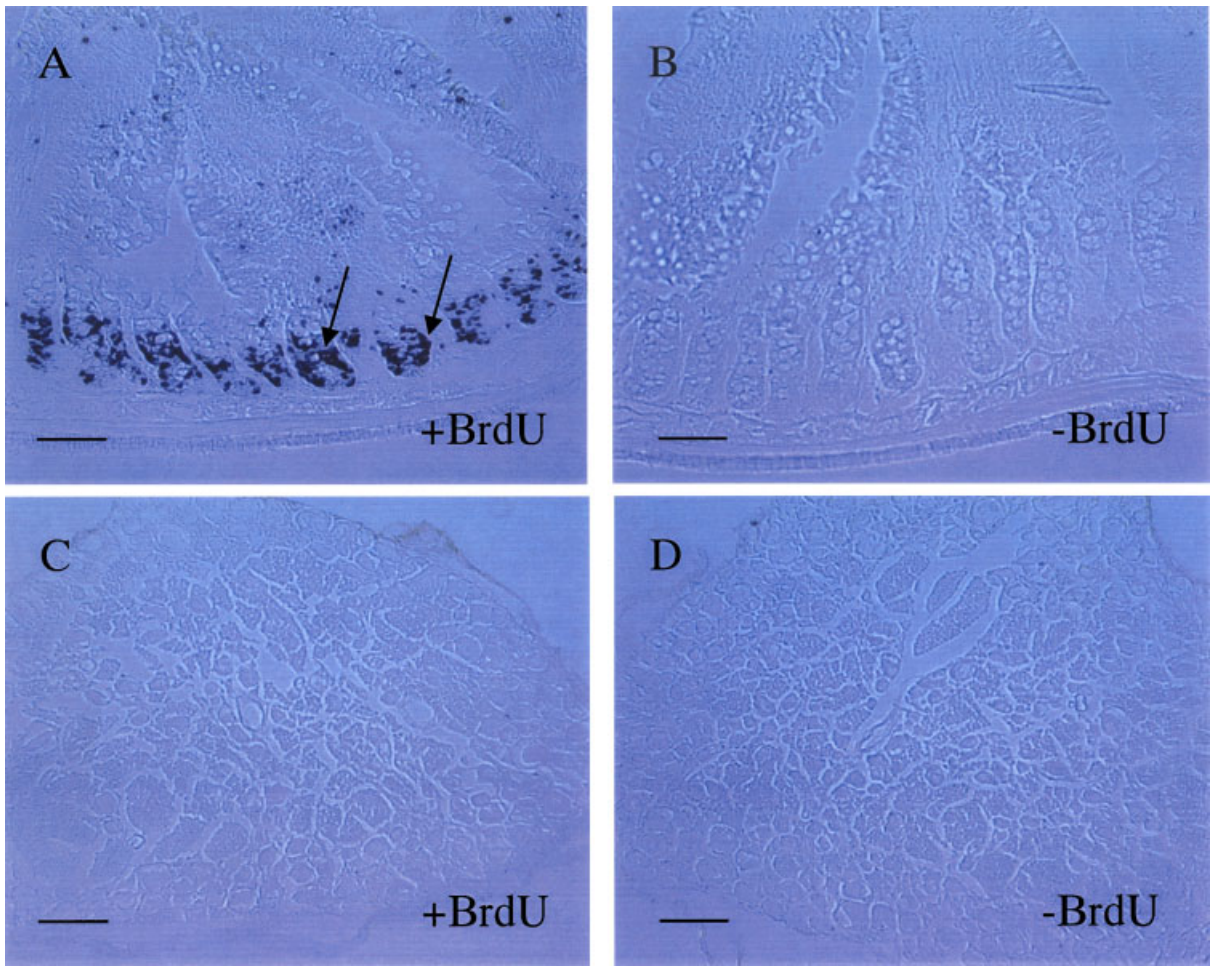

Fig. 3. Presence of BrdU staining in intestine (**A** and **B**) and its absence in regenerating and sham-operated dorsal root ganglia (**C** and **D**). A: In normal rat small intestine, BrdU staining is seen in a number of dividing cells in the crypts of the villi (arrows). B: Section from same animal as in (A), incubated in the absence of primary antibody. C, D: Absence of BrdU in sections of L5 DRG of experimental (C) and sham-operated (D) animals. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Reciprocally Regulated Gene Groups in Regenerating DRG Neurons, Activated Schwann Cells, and Activated Oligodendrocytes

Comparison of the accession numbers of genes up- or down-regulated in the three cell types revealed that there were groups of identical genes in DRG vs. Schwann, DRG vs. oligodendrocyte, and Schwann cell vs. oligodendrocyte that were differentially modulated. Shown in Table III, are the clusters of conserved genes showing reciprocal expression in the three cell types. In regenerating DRG neurons and activated Schwann cells, 113 or 54% of genes down-regulated in Schwann cells were identical to genes up-regulated in DRG neurons (Table III). Gene changes in DRG neurons and oligodendrocytes showed that a total of 53, equaling 75% of genes down-regulated in oligodendrocytes, were identical to genes up-regulated in DRG neurons. Gene changes in oligodendrocytes and Schwann cells showed that 23, equaling 32% of genes down-regulated in oligodendrocytes were identical to genes up-regulated in Schwann cells. Finally, there was a group of 22 genes that was common to all three gene groups, i.e., down-regulated in Schwann cells, down-regulated in oligodendrocytes, and up-regulated in DRG neurons. This group constituted 31% of down-regulated genes in oligodendrocytes (Table III). These relationships are shown diagramatically in Figure 4. This Venn diagram shows that the genes of

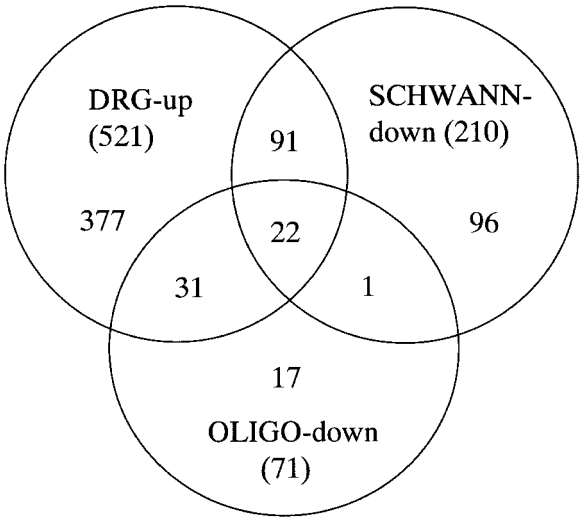

Fig. 4. Venn diagram indicating the reciprocal and coincident induction of identical genes in regenerating DRG cells, activated Schwann cells, and activated oligodendrocytes. Each circle contains 5 numbers. The numbers in brackets indicate the total number of genes modulated in each major category. Thus, 521 genes were up-regulated in DRG neurons, 210 genes were down-regulated in activated Schwann cells, and 71 were down-regulated in activated oligodendrocytes. The numbers that do not fall within an intersecting circle represent the number of genes that were not up or down-regulated in the other two cell types. Thus, 377, 96, and 17 genes were up-regulated in DRG neurons, and down-regulated in Schwann cells and oligodendrocytes, respectively. The numbers that fall within two intersecting circles represent identical genes (same accession number) that were either reciprocally or similarly regulated in the three tissue types. Thus, 91 genes that were up-regulated in DRG neurons were down-regulated in Schwann cells only, 31 genes that were up-regulated in DRG neurons were down-regulated in oligodendrocytes only, and 1 gene that was down-regulated in oligodendrocytes was also down-regulated in Schwann cells. Finally, 22 genes were up-regulated in DRG neurons and down-regulated in oligodendrocytes and down-regulated in Schwann cells (see also Table III).

common identity to each of the three tissues can be distributed into four categories, three of which overlap with the other two tissue types. These are: (i) genes specific to the individual cell type. Thus, 377 genes were up-regulated in DRG neurons, 96 were down-regulated in Schwann cells only and 17 were down-regulated in oligodendrocytes only; (ii) genes in common with either one of the other 2 cell types. In this category, 31 genes were reciprocally regulated specifically in DRG neurons and oligodendrocytes, and 91 genes were reciprocally regulated in DRG neurons and Schwann cells. Only one gene (LAMP Acc. no.: AA053501) was down-regulated that was common to both Schwann cells and oligodendrocytes, but not modulated in the regenerating DRG; (iii) genes that correlate with both of the other 2 cell types. In this group, there were 22 genes that were common to all three cell types (Fig. 4).

The known genes from each of these reciprocally regulated groups are shown in Table IV together with their differential expression values. Within the group of 22 genes expressed in all three cell types, there were 2 transcription factors, 1 cytokine receptor, 2 metabolic enzymes, 1 extracellular matrix protein, and 4 signaling proteins. The identity of the remaining 13 genes is unknown at the time of this writing. Within the group of genes specifically co-regulated in DRG neurons and Schwann cells, there were representatives from all cellular functions. However, there were proportionately more transcription factors (35%) and enzymes of anabolic metabolism (20%). In the group of genes specifically co-regulated in DRG neurons and oligodendrocytes, there were equal proportions of genes encoding secreted (25%) and signaling (25%) proteins (Table IV).

DISCUSSION

Nature of the Genetic Response in DRG neurons, Activated Schwann Cells, and Activated Oligodendrocytes

The experimental methods employed here have enabled the direct comparison of the genetic response of regenerating DRG neurons in vivo with activated Schwann cells and oligodendrocytes in vitro. It was found that 546, 452, and 79 genes were changed significantly in regenerating DRG neurons, LPS-treated Schwann cells, and LPS/IFN-γ-treated oligodendrocytes, respectively. Considering there were 10,000 genes on the chip, it is possible to predict that there is a significant change in the expression pattern of 4.5–5% of the genome of cells of the peripheral nervous system, when activated. This estimate is significantly less for glia from the CNS, where only 0.8% of this cell's genome was modulated when activated. In addition, it appears that distribution of the significantly changed genes varies among these three populations. Specifically, in the DRG population, 95% and 5% of significantly changed genes were up- or down-regulated, respectively, whereas in the Schwann cell population, 54 and 46% of the modulated genes were up- or down-regulated, respectively. In contrast to the

TABLE III. Clusters of Conserved Genes With Reciprocal Expression in Regenerating DRG Neurons, and Activated Schwann Cells, and Oligodendrocytes

	No. of genes	% of genes
		Schwann cell genes
Upreg. Sch and upreg. DRG	4	2
Upreg. Sch and downreg. DRG	4	2
Downreg. Sch and upreg. DRG	113	54
Downreg. Sch and downreg. DRG	0	0
		Oligodendrocyte genes
Upreg. oligo and upreg. DRG	1	1
Upregreg. oligo and downreg. DRG	0	0
Downreg. oligo and upreg. DRG	53	75
Downreg. oligo and downreg. DRG	0	0
		Oligodendrocyte genes
Upreg. oligo and upreg. Sch	0	0
Upreg. oligo and downreg. Sch	0	0
Downreg. oligo and upreg. Sch	0	0
Downreg. oligo and downreg. Sch	23	32
		Oligodendrocyte genes
Downreg. oligo/downreg. Sch/upreg. DRG	22	31
Downreg. oligo/downreg. Sch/downreg. DRG	0	0
Upreg. oligo/downreg. Sch/upreg. DRG	0	0
Upreg. oligo/downreg./Sch/downreg. DRG	0	0
Upreg. oligo/upreg. Sch/upreg. DRG	0	0
Upreg. oligo/upreg. Sch/downreg. DRG	0	0
Downreg. oligo/upreg. Sch/upreg. DRG	0	0
Downreg. oligo/upreg. Sch/downreg. DRG	0	0

Indicated are the numbers of identical (same accession number) genes that are differentially regulated in the three tissue types. Of all possible combinations, there is a strong correlation between genes that are down-regulated in Schwann cells and upregulated in DRG neurons, down-regulated in oligodendrocytes and up-regulated in DRG neurons, and down-regulated in both oligodendrocytes and Schwann cells (see also Fig. 4).

DRG-regulated genes, the activated oligodendrocytes, displayed a down-regulated genetic profile of 90% with only 10% of genes up-regulated. This may reflect an important difference between the glia of the peripheral and central nervous systems. The data may also reflect the activity of IFN-γ on oligodendrocytes, which is necessary to achieve an activation state in this cell type [Satoh et al., 1991].

The examination of purified, expanded Schwann cells and oligodendrocytes in vitro did ensure that the gene changes observed were related to one cell type. The examination of tissue in vivo could mean that the gene expression change observed could be related to more than one cell source, or caused by nearest neighbor tissues. In the case of the regenerating DRG, the observed gene changes could arise from neurons, the Schwann cells surrounding axons within the DRG or the satellite cells (similar to Schwann cells) which surround the neuronal cell bodies. However, considering the lesion site was in the mid-thigh, approximately 2 inches distal to the DRG, it is unlikely that other neurons or satellite cells were directly activated. Furthermore, the fact that there were 113 genes reciprocally regulated in DRG and activated Schwann cells suggests that the genes upregulated in the DRG are attributable to the axotomized neurons, rather than the surrounding Schwann cells.

Reciprocally Regulated Genes in DRG, Schwann Cells, and Oligodendrocytes

The main finding of this study is that components of the gene expression patterns of three different nervous system tissues (regenerating sensory neurons, activated Schwann cells, and activated oligodendrocytes) are: (a) identical and (b) reciprocally regulated. When looking at the modulation of identical genes between the three cell types (Table III), there was an apparent correlation in each case. These were: genes up-regulated in DRG neurons and down-regulated in Schwann cells (113 genes), genes up-regulated in DRG neurons and down-regulated in oligodendrocytes (53 genes), and genes down-regulated in Schwann cells and down-regulated in oligodendrocytes (23 genes). In addition there was a fourth group of genes composed of elements of the above three groups (22 genes) (Table III and Fig. 4).

Viewed from the perspective of a single cell type, for example DRG neurons, the genes fall

into four categories as shown in Figure 4. This finding of overlapping genes differentially regulated is similar to recent data that showed overlapping sets of genes in dendritic cells exposed to different stimuli [Huang et al., 2001]. Our results suggest that following peripheral axotomy, a large gene program, consisting of at least 521 genes is executed in DRG neurons. It appears that parts of this genetic program are identical to components or modules of gene programs that are executed specifically in LPS-activated Schwann cells (91 genes) or LPS/IFN-γ activated oligodendrocytes (31 genes). In addition, a smaller component consisting of 22 genes is identical to components of gene programs executed in both Schwann cells and oligodendrocytes (Fig. 4). It is possible to conclude that although the three cell types are phenotypically diverse, they share highly conserved gene programs that are executed upon environmental challenge. Furthermore, since only one gene was specifically shared between Schwann cells and oligodendrocytes, it would appear that activated myelinating cells of the central and peripheral nervous systems have more in common with regenerating DRG neurons than with each other. This latter finding may relate to the opposite responses of Schwann cells and oligodendrocytes following environmental stress to the PNS or CNS, respectively.

Why these genes were reciprocally regulated is unknown. However, it is possible that the highly conserved nature of their expression is a reflection of a general genotypic response for differentiation (DRG neurons) or proliferation (Schwann cells), each of which are mutually exclusive functions of mammalian cells. As an example, leukemia inhibitory factor (Table IV) causes the differentiation of neurons [Galli et al., 2000] and interphotoreceptor-retinoid-binding protein (up-regulated 13.4-fold) transports retinoic acid, a known neural differentiating agent [Stenkamp et al., 1998]. The membrane receptors for interferon alpha/beta [Kondo et al., 2000], ROR2 [Oishi et al., 1999], and TM4SF member SAS [Maecker et al., 1997] have also been shown to be associated with the differentiation of neurons and keratinocytes. Both NAB1 and KROX-20 are interdependent transcription factors that were previously shown to be involved in neural differentiation in the hindbrain [Mechta-Grigoriou et al., 2000]. Hypoxanthine phosphoribosyltransferase [Yeh et al., 1998], and glycinamide ribonucleotide synthetase [GARS, Brodsky et al., 1997], are necessary for synthesis of purines, which have been shown to have trophic effects in neurons and glia [Rathbone et al., 1999]. Semaphorin F (axon guidance-associated), gelsolin (neurite elongation-associated), [Westberg et al., 1999], and 14-3-3 isoforms [MacNicol et al., 2000] are clearly implicated in neural differentiation. ARL-3, upregulated 12-fold in this study, has been shown to bind cGMP phosphodiesterase, which is involved with differentiation of photoreceptor neurons in the retina [Colombaioni and Strettoi, 1993].

The up-regulation of these genes in de-differentiating (regenerating) neurons is consistent with their down-regulation in the LPS-treated Schwann cells and oligodendrocytes. As the proliferating glia were caused to differentiate to a bipolar or tripolar phenotype by the removal of 10% FBS and application of activation stimuli, it would be expected that proliferation-associated genes would be repressed (down-regulated) in these cells. Thus, the major function of the reciprocally regulated genes may be their involvement with cellular differentiation and change in phenotype, so as to survive in the presence of an inflammatory environment.

In relation to the finding of the subdivision of large gene programs into smaller components, it has recently been proposed that functional modules (composed of functionally related proteins), constitute a critical level of biological organization [Hartwell et al., 1999]. Modules have discrete functions within cells that arise from the interaction between their individual components, for example, the MAP-kinase signal transduction pathway. The possession of molecular modules, each with a discrete activity, confers on a cell a repertoire of highly conserved functions [Hartwell et al., 1999], that may be altered through the interaction between different modules. Using a model of cerebral ischemia, Jin et al. [2001], found the co-induction of several groups of related genes that might represent functional modules in the ischemic neuronal transcriptome. Thus, it is possible that the genetic program in activated neurons and glia of the peripheral/central nervous system may consist of a number of modules each of which encode a group of proteins. These proteins act in concert to perform a specific function [Hamilton et al., 2001] that is complimentary to those of other modules,

TABLE IV. Genes That Are Differentially Regulated in Regenerating DRG Neurons, Activated Schwann Cells, and Activated Oligodendrocytes

Category	Upreg. DRG	Downreg. Schwann	Downreg. oligo.
Nuclear			
VHL protein (W76188)	7.1	−2.2	−2
Geminin (N55327)	4	−2.4	−2.1
RAD52 (R96927)	2.5	—	−2.1
AH receptor (W67336)	10.4	—	−2.2
Helicase (AA001066)	2.1	−2	—
P53 protein (H61357)	4.1	−2.1	—
Mismatch repair (R41937)	2.5	−2.3	—
TATA box BP (W90373)	3.5	−2.3	—
DNA-bind. Prot. (W07182)	3.8	−2.4	—
RNA-dep prot. kin. (W42652)	5.4	−2.4	—
Krox-20 (AA018188)	2.4	−2.6	—
DNA-BP (N62901)	3.3	−2.6	—
Sp100 nuc. prot. (T50780)	2.9	−2.7	—
Orig. rec. comp. (H51719)	2.7	−3.2	—
DNA primase (N68268)	3.6	−3.3	—
NAB1 (T79325)	6.7	−3.4	—
Membrane			
IFN-R2 (R00872)	6.8	−2.6	−2.5
Anion channel (AA007636)	5.1	—	−2.1
LAMP (AA053501)	—	−2	−2
TNF rec. (H90177)	3	−2.1	—
ROR2 (AA024845)	4	−2.5	—
Comp. Rec. 1 (H75902)	5.2	−2.7	—
Semaphorin F (AA034415)	4.3	−3	—
SAS (W96199)	3	−3.1	—
Metabolism			
HPRT (W77728)	5.4	−3.7	−3.4
Cytochrome b (T63065)	3.5	−3.5	−2.1
DUDPase (AA058409)	2.1	—	−2.1
ATP synthase (W21200)	11.1	—	−2.3
UCP-3 (AA002183)	3.3	−2.1	—
Aspartoacyclase (N71653)	3	−2.1	—
Transacylase (R89083)	4.3	−2.2	—
Aminot'ferase (R19930)	3.2	−2.7	—
GART (N81002)	2.7	−3.1	—
Gal. trans'ase. (AA018766)	4.3	−2.3	—
Secreted			
RBP-3 (AA011047)	13.4	−3.3	−2.1
Leptin (R62951)	5.4	—	−2
NeuokininB (AA004764)	3.4	—	−2.1
Apolipoprot. A1 (AA004705)	2.4	—	−3.2
Selenoprotein P (N91230)	3.6	−2.5	—
LIF (R50354)	6	−2.9	—
Gelsolin (AA019754)	3.5	−3.8	—
Signaling			
WW-BP1 (AA041546)	13	−2	−3.1
Calgizzarin (R76776)	12.6	−2.6	−2.4
PI-3 kinase (AA042935)	8.4	−2.7	−2.6
ARL-3 (AA011142)	11.9	−3.1	−2
Beta 3-adaptin (N64530)	3	—	−2.5
Aden. kinase 5 (H09257)	6.4	—	−2.5
Ring finger prot. (R10283)	5.5	—	−2.4
Amyloid-BP1 (AA018451)	13	−2	—
Torsin 1B (AA058590)	2.3	−2.1	—
HSP-40 (N80249)	7.2	−2.6	—
14-3-3 beta (AA004530)	3.7	−2.6	—
WW-B. P (AA056053)	2.5	−2.7	—
Guanine NEF (W15542)	3.2	−3	—
Organele			
Kinesin 1B (W31909)	5.5	−3	−3.6
SNAP (N62961)	3.1	−4.4	−2.5
Mito. pep'ase (W31025)	3.7	—	−2.5
Apoptosis			
Caspase 6 (W44316)	2.1	−2.1	—

For each gene, the accession number and magnitude of change is indicated.

enabling the co-ordination of a complex cellular response. This response might be as general as ensuring differentiation or proliferation by the specifically effected cell [Hartwell et al., 1999].

Possible Role of DRG Gene Groups in the Regeneration Process: Cell Cycle Genes

It was observed that a number of genes associated with the regulation of the cell cycle, or with cancer cells (proliferating cells) were up-regulated in the DRG. Proliferation was ruled out however, after immunostaining for the mitotic marker BrdU was not observed in any sections of L4 or L5 DRG from nerve-crushed animals. Furthermore, the genes necessary for progression through the cell cycle (cyclin A, cyclin B1, Cdc2, Cdc26, Cdc28, Cdk 2, Cdk 4), were not present in the group of genes regulated by a factor of ± 2.0 in either DRG or Schwann cells, though they were represented on the chip. This finding suggests that these genes may perform some function in the regenerating neurons unrelated to mitosis. This may include a co-ordination of the general response of increased RNA synthesis [Wells and Vaidya, 1994] of the axotomized neurons. Indeed, it has been shown that cell cycle regulation genes are expressed in normal hippocampal neurons and in hippocampal neurons which survive an ischemic episode, suggesting a function other than the induction of mitosis [Jin et al., 2001].

Embryonic neurons display a normal cell cycle, for the purpose of cell division and generation of neuronal progeny. However, we propose the existence of a "regeneration cycle" in adult DRG neurons following a crush injury. In this case, the genetic program activating RNA and protein synthesis is for the purpose of cell survival, axonal elongation and reconstitution of connections. It is possible that control of the regeneration cycle in adult neurons depends on the deployment of a small subset of genes which, during embryogenesis, were concerned with mitosis and the generation of neuronal progenitors. In the adult animal, these genes are regulated as part of a large-scale induction of transcription in the absence of DNA replication for the purpose of regeneration.

Possible Role of DRG Gene Groups in the Regeneration Process: Signaling Genes

Recent studies indicate that signaling events mediated by MAP kinase or GTP-associated proteins [Tanabe et al., 2000] are critical for regeneration in DRG neurons. Figure 2A,B shows the genes encoding proteins known to be associated with GTP-binding proteins and the MAP-kinase signaling pathway, respectively, that were shown to be up-regulated by the array. Firstly, there are those genes encoding proteins associated with members of the super-family of GTP-binding proteins (small G-proteins) with intrinsic GTPase activity, and which cycle between GTP- and GDP-bound states (Fig. 2A). These proteins act as molecular switches that activate a variety of secondary pathways, resulting in alterations of cell phenotype [Scita et al., 2000]. The proteins induced in DRG neurons in this category, are upstream regulators or docking/adapter proteins for a number of GTP-ases previously shown to be instrumental in neuronal regeneration [Gallo and Letourneau, 1998; Kozma et al., 1995]. For example, Radixin, induced 2.0-fold, is a downstream effector of Rho-GTP [Mackay et al., 1997], and is involved in growth cone stability and motility [Gonzalez-Agosti and Solomon, 1996]. IQGAP2, induced 5-fold, is an upstream regulator for the Rho homolog CDC42 [McCallum et al., 1996], which is involved in the cytoskeletal remodeling that occurs during neuronal dendritic development [Threadgill et al., 1997]. ARL-3 is a member of the ARF-GTP binding protein family [Cavenagh et al., 1994], and may interact with ARF (adenosine ribosylation factor) proteins [Cavenagh et al., 1994] which have recently been shown to be present in neuronal growth cones [Hess et al., 1999]. Secondly, there is induction of those genes encoding proteins associated with the MAP-kinase signaling pathway (Fig. 2B). They are SH3BP2 and GAB2, which are adapter proteins, and which facilitate the interaction between phosphatidylinositol kinase-3 and receptor tyrosine kinases [Bouillie et al., 1999; Gadina et al., 2000]. A study by Gadina et al. [2000] showed that phosphorylation of GAB2 correlated with activation of the MAP-kinase pathway. Another component of the MAP-kinase pathway, 14-3-3 beta, was induced 4-fold. This protein is an essential co-factor for c-Raf, an enzyme downstream of Ras-GTP, with which it forms a heterodimer [Tzivion et al., 1998]. 14-3-3 isoforms have been shown to be up-regulated in regenerating hypoglossal motorneurons in the rat [Namikawa et al., 1998]. Although c-Raf was not changed on the microarray, a closely related enzyme, Cot/Tpl2 was induced 3-fold.

This enzyme is an activator of MAPK-kinase (MEK), and has been shown to induce neurite outgrowth in PC12 cells [Hagemann et al., 1999]. Finally, Gs alpha, the stimulatory subunit of the heterotrimeric GTP-binding protein, and which also activates MAP-kinases via protein kinase C [Han and Conn, 1999], was induced 4-fold (Fig. 2B). Taken together, the induction of these genes in the present study confirms recent studies which show that neuronal growth and survival is dependent upon signaling through the phosphatidyl-inositol-3 kinase and Ras-MAP kinase pathway [Mihaly et al., 1996; Namikawa et al., 1998; Encinas et al., 1999; Atwal et al., 2000; Schmidt et al., 2000; Kimpinski and Mearow, 2001].

Additional studies are needed to determine the functions of the groups of reciprocally regulated genes in regenerating neurons, activated Schwann cells, and activated oligodendrocytes. The application of cluster analysis and other statistical methods [Somogyi and Sniegoski, 1996; Wen et al., 1997] to data obtained at multiple time points during regeneration or activation, should further clarify the mechanism of the genetic programs invoked in these three cell types. Furthermore, future microarrays containing the entire human gene repertoire will be available for a complete genetic response and analysis.

REFERENCES

Atwal JK, Massie B, Miller FD, Kaplan DR. 2000. The TrkB-Shc site signals neuronal survival and local axon growth via MEK and PI3-kinase. Neuron 27:265–277.

Bouillie S, Barel M, Frade F. 1999. Signaling through the EBV/C3d receptor (CR2, CD21) in human B lymphocytes: Activation of phosphatidylinositol 3-kinase via a cd19-independent pathway. J Immunol 162:136–143.

Brockes JP, Fields KL, Raff MC. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res 165: 105–118.

Brodsky G, Barnes T, Bleskan J, Becker L, Cox M, Patterson D. 1997. The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome. Hum Mol Genet 6:2043.

Cajal SR. 1928. Degeneration and regeneration in the nervous system. London: Oxford University Press.

Caroni P, Schwab ME. 1988. Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white mater. Neuron 1:85–96.

Cavenagh MM, Breiner M, Schurmann A, Rosenwald AG, Terui T, Zhang C-J, Randazzo PA, Adams M, Joost HG, Khan RAS. 1994. ADP-ribosylation factor (ARF)-like 3, a new member of the ARF family of GTP-binding proteins cloned from human and rat tissues. J Biol Chem 269: 18937–18942.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME. 2000. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403:434–439.

Colombaioni L, Strettoi E. 1993. Appearance of cGMP-phosphodiesterase immunoreactivity parallels the morphological differentiation of photoreceptor outer segments in the rat retina. Vis Neurosci 10:395–402.

Encinas M, Iglesias M, Llecha N, Comella JX. 1999. Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y. J Neurochem 73: 1409–1421.

Farlow D, Vansant G, Cameron AA, Chang JJ, Khoh-Reiter S, Pham N-L, Sagara Y, Carlo DJ, Ill CR. 2000. Gene expression monitoring for gene discovery in models of peripheral and central nervous system differentiation, regeneration and trauma. J Cell Biochem 80:171–180.

Fu SY, Gordon T. 1997. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14:67–116.

Gadina M, Sudarshan C, Visconti R, Zhou YJ, Gu H, Neel BG, O'Shea JJ. 2000. The docking protein gab2 is induced by lymphocyte activation and is involved in signaling by interleukin-2 and interleukin-15 but not other common gamma chain-using cytokines. J Biol Chem 275:26959–26966.

Galli R, Pagano SF, Gritti A, Vescovi AL. 2000. Regulation of neuronal differentiation in human CND stem cell progeny by leukemia inhibitory factor. Dev Neurosci 22: 86–95.

Gallo P, Letourneau PC. 1998. Axon guidance: GTPases help axons reach their targets. Curr Biol 8:R80–R82.

Giehl KM, Tetzlaff W. 1996. BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. Eur J Neurosci 8:1167–1175.

Gonzalez-Agosti C, Solomon F. 1996. Response of radixin to perturbations of growth cone morphology and motility in chick sympathetic neurons in vitro. Cell Motil Cytoskel 34:122–136.

Groves MJ, Ng YW, Ciradi A, Scaravilli F. 1996. Sciatic nerve injury in the adult rat: Comparison of effects on oligodaccharide, CGRP and GAP-43 immunoreactivity in primary afferents following two types of trauma. J Neurocytol 25:219–231.

Hagemann D, Troppmair J, Rapp UR. 1999. Cot proto-oncogene activates the dual specificity kinases MEK-1 an SEK-1 and induces differentiation of PC12 cells. Oncogene 18:1391–1400.

Hamilton M, Liao J, Cathcart MK, Wolfman A. 2001. Constitutive association of c-N-Ras with c-Raf-1 and protein kinase C epsilon in latent signaling modules. J Biol Chem 276:29079–29090.

Han XB, Conn PM. 1999. The role of protein kinases A and C in the regulation of mitogen-activated protein protein kinase activation in response to gonadotropin-releasing hormone receptor activation. Endocrinology 140:2241–2251.

Hartwell LJ, Hopfield JJ, Leibler S, Murray AW. 1999. From molecular to modular biology. Nature 402(Suppl): C47–C52.

Hess DT, Smith DS, Patterson SI, Khan RA, Pate-Skene JH, Norden JH. 1999. Rapid arrest of axon elongation by Brefeldin A: A role for the small GTP-binding protein ARF in neuronal growth cones. J Neurobiol 38:105–115.

Hewett JA, Hewett SJ, Winkler S, Pfeiffer S. 1999. Inducible nitric oxide synthase expression in cultures enriched for mature oligodendrocytes is due to microglia. J Neurosci Res 56:189–198.

Hirata K, Mitoma H, Ueno N, He JW, Kawabuchi M. 1999. Differential response of macrophage subpopulations to myelin degradation in the injured rat sciatic nerve. J Neurocytol 28:685–695.

Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA, Lander ES, Hacohen N. 2001. The plasticity of dendritic cell responses to pathogens and their components. Science 294:870–875.

Jacob JM, McQuarrie IG. 1996. Assembly of microfilaments and microtubules from axonally transported actin and tubulin after axotomy. J Neurosci Res 43:412–419.

Jin K, Mao XO, Eshoo MW, Nagayama T, Minami M, Simon RP, Greenberg DA. 2001. Microarray analysis of hippocampal gene expression in global cerebral ischemia. Ann Neurol 50:93–103.

Kimpinski K, Mearow K. 2001. Neurite growth promotion by nerve growth factor and insulin-like growth factor-1 in cultured adult sensory neurons: Role of phosphoinositide 3-kinase and mitogen activated protein kinase. J Neurosci Res 63:486–499.

Kondo M, Nagano H, Sakon M, Yamamoto H, Morimoto O, Arai I, Miyamoto A, Eguchi H, Dono K, Nakamori S, Umeshita K, Wakasa K, Ohmoto Y, Monden M. 2000. Expression of interferon alpha/beta receptor in human hepatocellular carcinoma. Int J Oncol 17:83–88.

Kozma R, Ahmed S, Best A, Lim L. 1995. The ras-related protein CDC42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15:1942–1952.

Lander ES, et al. 2001. Initial sequencing and analysis of the human genome. Nature 409:860–921.

Leah JD, Herdegen T, Bravo R. 1991. Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: Evidence for a role in the regeneration process. Brain Res 566:198–207.

Mackay DJ, Esch F, Furthmayr H, Hall A. 1997. Rho- and Rac-dependent assembly of focal adhesion complexes and actin fillaments in permeabilized fibroblasts: An essential role for ezrin/radixin/moesin proteins. J Cell Biol 138:927–938.

MacNicol MC, Muslin AJ, MacNicol AM. 2000. Disruption of the 14-3-3 binding site within the B-Raf kinase domain uncouples catalytic activity from PC12 cell differentiation. J Biol Chem 275:3803–3809.

Maecker HT, Todd SC, Levy S. 1997. The tetraspanin superfamily: Molecular facilitators. FASEB J 11:428–442.

McCallum SJ, Wu WJ, Cerione RA. 1996. Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. J Biol Chem 271:21732–21737.

Mechta-Grigoriou F, Garel S, Charnay P. 2000. Nab proteins mediate a negative feedback loop controlling KROX-20 activity in the developing hindbrain. Development 127:119–128.

Mihaly A, Priestly JV, Molnar E. 1996. Expression of raf serine/threonine protein kinases in the cell bodies of primary sensory neurons of the adult rat. Cell Tiss Res 285:261–271.

Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P. 2000. Molecular characterizaion of schizophrenia viewed by microarray analysis of gene expression in the prefrontal cortex. Neuron 28:53–67.

Moalem G, Gdalyahu A, Shani Y, Otten U, Lazarovici P, Cohen IR, Schwartz M. 2000. Production of neurotrophins by activated T cells: Implications for neuroprotective autoimmunity. J Autoimmun 15:331–345.

Montgomery CT, Tenaglia EA, Robson JA. 1996. Axonal growth into tubes within lesions in the spinal cords of adult rats. Exp Neurol 137:277–290.

Namikawa K, Su Q, Kiru-Seo S, Kiyama H. 1998. Enhanced expression of 14-3-3 family members in injured motoneurons. Mol Brain Res 55:315–320.

Oishi IS, Takeuchi R, Hashimoto A, Nagabukuro T, Ueda ZJ, Liu T, Hatta S, Akira Y, Matsuda H, Yamamamura H, Otani Y. 1999. Spatio-temporally regulated expression of receptor tyrosine kinases, mROR1, mROR2 during mouse development: Implications in development and function of the nervous system. Genes Cells 4:41–56.

Perry GW, Wilson DL. 1981. Protein synthesis and axonal transport during nerve regeneration. J Neurochem 37: 1203–1217.

Podhajsky RJ, Myers RR. 1993. The vascular response to nerve crush: Relationship to Wallerian degeneration and regeneration. Brain Res 623:117–123.

Rathbone MP, Middelmiss PJ, Gysbers JW, Andrew C, Herman MA, Reed JK, Ciccarelli R, DiIorio P, Caciagli F. 1999. Trophic effects of purines in neurons and glial cells. Prog Neurobiol 59:663–690.

Salonen V, Aho H, Royotta M, Peltonen J. 1988. Quantitation of Schwann cells and endoneurial fibroblast-like cells after experimental nerve trauma. Acta Neuropathol 75:331–336.

Satoh J, Kastrukoff LF, Kim SU. 1991. Cytokine-induced expression of intercellular adhesion molecule-1 (ICAM-1) in cultured human oligodendrocytes and astrocytes. J Neuropathol Exp Neurol 50:215–226.

Schena M, Shalon D, Davis RW, Brown PO. 1995. Quantitative monitoring of gene expression patterns with a complimentary DNA microarray. Science 270: 467–470.

Schmidt RS, Pruitt WM, Maness PF. 2000. A MAP kinase-signaling pathway mediates neurite outgrowth on L1 and requires Src-dependent endocytosis. J Neurosci 20:4177–4188.

Scita G, Tenca P, Frittoli E, Toccetti A, Innocenti M, Giardina G, di Firoe PP. 2000. Signaling from Ras to Rac and beyond:not just a matter of GEFs. EMBO J 19:2393–2398.

Shuman SL, Bresnahan JC, Beattie MS. 1997. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. J Neurosci Res 50:798–808.

Skundric DS, Bealmear D, Lisak RP. 1997. Induced upregulation of IL-1, IL-1RA, and IL-1R type I gene expression by Schwann cells. J Neuroimmunol 74:9–18.

Somogyi R, Sniegoski CA. 1996. Modeling the complexity of genetic networks: Understanding multigenic and pleotropic regulation. Complexity 1:45–63.

Stenkamp DL, Cunningham LL, Raymmond PA, Gonzalez-Fernandez F. 1998. Novel expression pattern of interphotoreceptor retinoid-binding protein (IRBP) in the adult and developing zebrafish retina and RPE. Mol Vis 4:26.

Tanabe K, Tachibana T, Yamashita T, Che YH, Yoneda Y, Ochi T, Tohyama M, Yoshikawa H, Kiyama H. 2000. The small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats. J Neurosci 20:4138–4144.

Threadgill R, Bobb K, Ghosh A. 1997. Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. Neuron 19:625–634.

Toews AD, Barrett C, Morell P. 1998. Monocyte chemoattractant protein is responsible for macrophage recruitment following injury to sciatic nerve. J Neurosci Res 53:260–267.

Tseng GF, Prince DA. 1996. Structural and functional alterations in rat corticospinal neurons after axotomy. J Neurophysiol 75:248–267.

Tzivion G, Luo Z, Avruch J. 1998. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. Nature 394:88–92.

van der Pal RH, Vos JP, van Golde LM, Lopes-Cardozo M. 1990. A rapid procedure for the preparation of oligodendrocyte-enriched cultures from rat spinal cord. Biochim Biophys Acta 1051:159–165.

Venter JC, et al. 2001. The sequence of the human genome. Science 29:1304–1351.

Vilar MJ, Cortes R, Theodorsson E, Wiesenfeld-Hallin Z, Schalling M, Fahrenkrug J, Emson PC, Hokfelt T. 1989. Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. Neurosci 33:587–604.

Wells MR, Vaidya U. 1994. RNA transcription in axotomized dorsal root ganglion neurons. Brain Res 27:163–166.

Wen X, Fuhrman S, Michaels GS, Carr DB, Smith S, Barker JL, Somogyi R. 1997. Large-scale temporal gene expression mapping of central nervous system development. Proc Nat Acad Sci USA 95:334–339.

Westberg JA, Zhang KZ, Andersson LC. 1999. Regulation of neural differentiation by normal and mutant (G654A, amuloidogenic) gelsolin. FASEB J 13:1621–1626.

Yeh J, Zheng S, Howard BD. 1998. Impaired differentiation of HPRT-deficient dopaminergic neurons: A possible mechanism underlying neuronal dysfunction in Lesch-Nyhan syndrome. J Neurosci Res 53:78–85.

Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M, Johnston R. 2001. An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. Nucleic Acids Res 29:E41–E51.