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EGR2 Mutations in Inherited Neuropathies Dominant-Negatively Inhibit Myelin Gene Expression

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Summary

The identification of *EGR2* mutations in patients with neuropathies and the phenotype *Egr2/Krox20*$^{-/-}$ have demonstrated that the Egr2 transcription factor is critical for peripheral nerve myelination. However, the mechanism by which these mutations cause disease remains unclear, as most patients present with disease in the heterozygous state, whereas *Egr2*$^{+/-}$ mice are phenotypically normal. To understand the effect of aberrant Egr2 activity on Schwann cell gene expression, we performed microarray expression profiling to identify genes regulated by Egr2 in Schwann cells. These include genes encoding myelin proteins and enzymes required for synthesis of normal myelin lipids. Using these newly identified targets, we have shown that neuropathy-associated *EGR2* mutants dominant-negatively inhibit wild-type Egr2-mediated expression of essential myelin genes to levels sufficiently low to result in the abnormal myelination observed in these patients.

Introduction

Axonal myelination by oligodendrocytes in the central nervous system (CNS) and by Schwann cells in the peripheral nervous system (PNS) is crucial for saltatory conduction and thus proper signal propagation. Demyelinating diseases such as the hereditary motor and sensory neuropathies (HMSN) in the PNS and multiple sclerosis in the CNS cause severe motor and sensory deficits resulting in significant patient morbidity and mortality. In fact, HMSN-1, classically referred to as hypertrophic Charcot-Marie-Tooth Disease, is one of the most common genetic disorders, affecting one person in 2500 (Lupski, 1997). Mutations in several known myelin genes, myelin protein zero (*MPZ*), peripheral myelin protein 22 (*PMP22*), connexin 32 (*Cx32*), and *periaxin*, have been identified in patients with one of several inherited peripheral neuropathies including Charcot-Marie-Tooth and Dejerine-Sottas Syndrome (Boerkoel et al., 2001; Guilbot et al., 2001; Lupski et al., 1991; De Jonghe et al., 1997; Raeymaekers et al., 1991). However, a significant portion of patients with inherited myelinopathies do not have mutations in myelin genes, leading to the hypothesis that mutations in other genes vital for normal peripheral nerve myelination were responsible for causing disease in these patients.

The *EGR2* gene became a candidate for mutation analysis in these patients because of results obtained in studies of mice containing mutations in this transcription factor. While mice heterozygous for an *Egr2* (also known as *Krox20*) null allele are phenotypically normal, *Egr2*-deficent mice have severely defective myelination of peripheral nerves. This defect is characterized by Schwann cells that become arrested after having formed a one-to-one relationship with axons and that express very low levels of MPZ and myelin basic protein (MBP) (Topilko et al., 1994). Indeed, sequence analyses conducted by several groups revealed that some patients with inherited peripheral neuropathies harbor mutations in the *EGR2* gene, predominantly in the DNA binding domain (DBD) (Bellone et al., 1999; Botti et al., 1998; Timmerman et al., 1999; Warner et al., 1998). Because *Egr2* heterozygous mice are phenotypically normal, it was surprising that the disease followed a dominant mode of inheritance in families with neuropathy caused by *EGR2* DBD mutations. In fact, subsequent gel shift assays and results from transfected luciferase reporter constructs demonstrated that the EGR2 DBD mutants lacked DNA binding and transcriptional activity (Warner et al., 1999). Taken together, these observations led to the hypothesis that the DBD mutations in *EGR2* do not generate simple loss-of-function alleles, but that these mutant alleles behave dominantly to disrupt wild-type Egr2 function.

In this study, we combine several powerful new technologies to prove that the DBD mutant Egr2 proteins dominantly inhibit wild-type induction of critical myelin genes. First, using microarray expression analysis on a genome wide scale after quantitative Schwann cell infection with *Egr2*-expressing adenoviruses, we show that Egr2 is sufficient for induction of genes critical for myelin formation and maintenance. These include genes encoding the major myelin proteins, MPZ, PMP22, MBP, myelin associated glycoprotein (MAG), Cx32, and periaxin. Furthermore, genes encoding enzymes essential for the synthesis of lipids and cholesterol, which are critical components of peripheral myelin, are also induced by *Egr2*. Second, by monitoring Schwann cell expression of these newly identified Egr2 target genes from their endogenous chromosomal loci, we demonstrate that the EGR2 mutants from patients with dominantly inherited peripheral neuropathies effectively inhibit wild-type Egr2-mediated Schwann cell expression of essential myelin genes. These results indicate that the peripheral myelinopathy observed in patients harboring DBD mutant *EGR2* alleles can be explained by the dominant-negative inhibition of Egr2-mediated myelin gene expression. Finally, we show that introduction of Egr2 into postnatal, *Egr2*-deficient Schwann cells results in expression of genes critical for myelination, suggesting that *EGR2* mutant Schwann cells found in patients with

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inherited peripheral neuropathies may be capable of myelinating axons in response to reintroduction of wild-type Egr2.

Results

Neuropathy-Associated *Egr2* DNA Binding Domain Mutants Do Not Function as Dominant Negative Inhibitors on Synthetic Promoter-Reporter Constructs

EGR2 mutations have been found in patients with inherited peripheral myelinopathy, such as congenital hypomyelinating neuropathy (CHN) or Charcot-Marie-Tooth (CMT). Most of these mutations are located within the DNA binding domain (DBD) and exhibit greatly decreased (or absent) DNA binding and transcriptional activity (Warner et al., 1998; Warner et al., 1999). From these results, they appear to be simple loss-of-function alleles that would be expected to cause disease in a recessively inherited manner. Consistent with this hypothesis, mice which are heterozygous for a null *Egr2* allele are phenotypically normal (Murphy et al., 1996). However, all of the *EGR2* DBD mutant alleles cause disease in the heterozygous state (i.e., they all display a dominant mode of inheritance). Thus, it has been hypothesized that the DBD mutations in *EGR2* are acting as dominant-negative alleles (Warner et al., 1999).

Mutations in the DBD of the Wilms' tumor gene product (WT-1), associated with Denys-Drash syndrome, or in p53, associated with Li-Fraumeni syndrome, have been characterized. Using transfection experiments with reporter gene constructs containing synthetic promoters composed of either WT-1 or p53 binding sites, it was shown that these mutant transcription factors act as dominant-negative inhibitors, as they inhibit transcriptional activation by their wild-type counterpart in a dose-dependent fashion (Aurelio et al., 2000; Reddy et al., 1995; Saifudeen et al., 2000). Using an analogous approach, we tested the Egr2(S382R, D383Y) mutant, which causes a severe peripheral neuropathy in the heterozygous state, for dominant-negative activity. We cotransfected expression constructs of wild-type *Egr2* and the *Egr2(S382R, D383Y)* mutant in varying amounts, along with a reporter plasmid containing four Egr2 binding sites upstream of the luciferase gene, into CV-1 cells. We found no evidence of dominant-negative activity using the Egr2 DBD mutant, even at ratios as high as 4:1 (Figure 1A). We reasoned that the apparent lack of dominant-negative activity might be due to an inappropriate cell context. Thus, we conducted similar transfection experiments using wild-type *Egr2* and *Egr2(S382R, D383Y)* with the Egr2 binding site reporter plasmid in rat Schwann cells. Again, we were unable to observe any significant dominant-negative activity of Egr2(S382R, D383Y) (Figure 1B). To determine whether the failure to observe dominant-negative activity was due to an inappropriate promoter context, we cotransfected wild-type *Egr2* and *Egr2(S382R, D383Y)* along with a reporter plasmid containing 1.3 kb of the MPZ promoter, which can be activated by Egr2 (Zorick et al., 1999), into rat Schwann cells. Once again, even at ratios as high as 4:1, no notable dominant-negative activity was observed (Figure 1C).

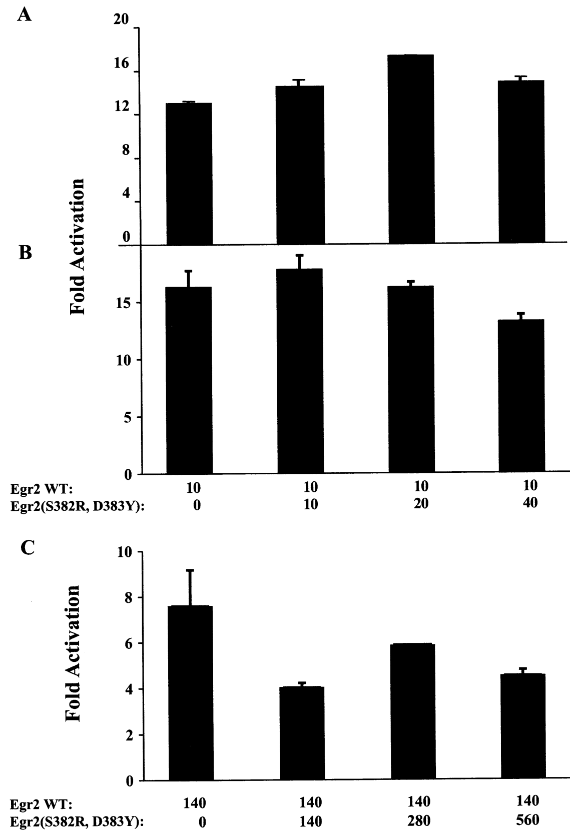


Figure 1. Synthetic Promoter-Reporter Constructs Do Not Reveal a Dominant-Negative Function for Egr2 DNA Binding Domain Mutants

(A) CV-1 cells were transfected with a luciferase reporter construct containing four Egr1 binding sites upstream of the prolactin minimal promoter and the indicated amounts of either wild-type or mutant (S382R,D383Y) Egr2.

(B) Rat Schwann cells were transfected with the same constructs as in (A).

(C) Rat Schwann cells were transfected with a luciferase reporter construct containing 1.3 kb of the *MPZ* promoter and the indicated amounts of either wild-type or mutant (S382R,D383Y) Egr2. The scale indicates the fold activation of the reporter by Egr2.

Identification of *Egr2* Target Genes by Microarray Analysis

The dominant inheritance pattern of the neuropathy observed in families harboring EGR2 DBD mutant was inconsistent with our inability to demonstrate dominant-negative activity in the transfection assays. One potential explanation for this discrepancy was the inability of the promoter-reporter constructs to adequately recapitulate Egr2-mediated regulation of relevant target promoters in their native chromosomal loci. Therefore, to elucidate the mechanism by which a single copy of these mutant *EGR2* alleles results in myelinopathy, it became apparent that the identification of genes regulated by Egr2 in Schwann cells was necessary. This would allow us to test the effects of the DBD mutant Egr2 proteins on the transcriptional regulation of specific Egr2 target genes from their endogenous chromosomal loci.

To identify target genes, we employed a gain-of-function model in which Egr2 is expressed in the absence of stimuli that might also induce other transcription factors

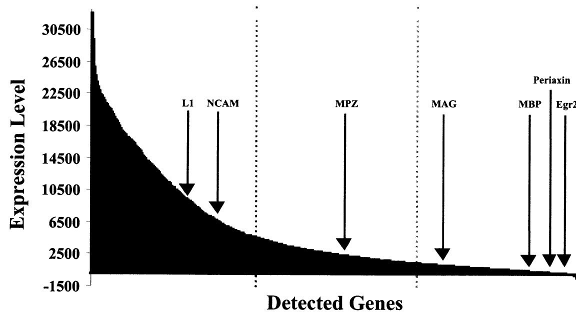


Figure 2. Myelinating Schwann Cell Markers Are Expressed at Low Levels in Cultured Schwann Cells

The expression level of each detected gene from the control sample (cultured Schwann cells infected with EGFP-expressing adenovirus) was plotted in decreasing order. Dotted gray lines categorize each gene as being expressed in upper, middle, or lower third of all genes represented on the GeneChip. Nonmyelinating Schwann cell markers, L1 and NCAM, fell in the upper third, indicating high-level mRNA expression. Conversely, genes encoding the myelin proteins, MPZ, MAG, periaxin, and MBP, as well as the transcription factor Egr2 were found mainly in the lower third, implying that these genes are expressed at low levels in cultured Schwann cells.

involved in peripheral nerve myelination. To this end, we used adenoviral-mediated expression of EGFP (control) or Egr2 in primary rat Schwann cells and conducted oligonucleotide microarray expression analysis (Rat Genome U34 Array Set, Affymetrix [Santa Clara, CA], containing a total of containing a total of 7000 known genes). Analysis of the expression profile of Schwann cells infected for 24 hr with the EGFP expressing adenovirus (control) revealed that the expression level of *Egr2* in primary Schwann cells was very low (Figure 2), suggesting that potential Egr2 target genes were not expressed prior to adenoviral-mediated expression of Egr2. This analysis also showed that mRNA levels of the nonmyelinating Schwann cell markers, L1 and neural cell adhesion molecule (NCAM), were among the top 25% of all expressed genes, whereas genes encoding myelin proteins, MPZ, periaxin, MBP, and MAG, were in the lower 50% of all expressed genes (Figure 2). This expression profile demonstrated that cultured Schwann cells exhibit many of characteristics associated with premyelinating or nonmyelinating Schwann cells, and they would therefore serve as a good model for identifying Egr2 target genes.

To identify genes induced by Egr2 expression, we then conducted microarray expression analysis of Schwann cells infected with the Egr2-expressing adenovirus for 24 hr. The average hybridization intensity across all probe sets from the "Egr2" microarray was normalized to that of the "EGFP" (control) microarray. Of the genes found to be "present" or "increased" on the GeneChip analysis, we selected genes that were induced more than 3-fold for further analysis. These 98 known genes, comprising ~1.3% of all genes represented on the array, were placed in functional categories (Table 1). Three genes strongly expressed in Schwann cells, MAG (22.4-fold; rank 6th), periaxin (10-fold; rank 20th), and MPZ (6.2-fold; rank 49th) were highly induced by Egr2. In addition, several genes, encoding enzymes essential for lipid and cholesterol synthesis, and two splice variants of VEGF and their receptor flk-1, were also induced by Egr2.

Egr2 Induces Expression of Critical Myelin Genes

To verify the induction of these target genes, mouse Schwann cells were infected with control or Egr2 expressing adenovirus for 24 hr, and quantitative RT-PCR was performed. While expression of the pan Schwann cell marker S100β (1.08-fold) and of the nonmyelinating Schwann cell markers L1 (0.5-fold) and NCAM (1.4-fold) were largely unaffected, mRNA levels of genes characteristic of myelinating Schwann cells, MAG (10.8-fold), periaxin (12.6-fold), MPZ (59.2-fold), PMP22) (15.8-fold), and Cx32 (8.6-fold), were greatly increased by Egr2 (Figure 3). Mouse Schwann cells were used for the remainder of our studies as the dynamic range of target gene induction proved to be greater in these cells. For example, the basal expression (i.e., in control infected Schwann cells) of MPZ in mouse Schwann cells is much lower than in rat Schwann cells (data not shown), resulting in a greater relative induction of this mRNA in the mouse system.

Several studies have shown that MBP is a late Schwann cell marker, and its rise in expression in postnatal development follows that of the myelinating Schwann cell markers (Notterpek et al., 1999). For example, levels of MBP protein reach half maximal levels on postnatal day 6, lagging behind MPZ and PMP22, which are already at half maximal levels by postnatal day 3. Indeed, while Egr2 did not induce MBP in the first 24 hr after adenoviral infection, when its mRNA levels were examined 48 hr after adenoviral-mediated Egr2 expression, MBP was induced 6.2-fold (Figure 3). These results indicate that Egr2 activates expression of six genes characteristic of myelinating Schwann cells and suggest that other intermediate steps may be required for Egr2-mediated expression of MBP.

Because these target genes were identified using Schwann cells cultured in vitro, we also analyzed their expression following peripheral nerve injury, an in vivo paradigm where Egr2 is dynamically regulated. After nerve crush, *Egr2* expression dramatically declines shortly after the injury and then gradually recovers as the nerve remyelinates (Zorick et al., 1996). The sciatic nerves of anesthetized adult rats were crushed using forceps, and the distal segment of these injured nerves was harvested at multiple time points for quantitative RT-PCR analysis. Consistent with previous results, we found that *Egr2* expression was decreased for 7–14 days following sciatic nerve crush (Figure 4A). The expression of the myelinating Schwann cell markers (MPZ, MAG, Periaxin, and MBP) paralleled this decrease during the first two weeks after nerve crush and their recovery was delayed relative to *Egr2* (Figure 3A). In addition to the myelin proteins, we profiled the expression of several other newly identified Egr2 target genes, including VEGF3, PTHrP, Nab2 (NGFI-A binding protein 2), and AA866443, an EST with no homology to known genes. Expression analysis of these four genes after nerve injury yielded a profile similar to that of the myelin proteins (Figure 4B). Thus, genes identified as Egr2 target genes in an in vitro paradigm are expressed in the Schwann cells of an injured peripheral nerve in a pattern consistent with regulation by Egr2 in vivo.

Table 1. Broad Functional Characterization of Egr2 Responsive Genes in Schwann Cells

Accession Number	Gene	Fold Induction
	Membrane Proteins	
AF023302	Reggie1-1	25.4
X96394	MDR	9
AF086758	Na-K-2Cl cotransporter	7.8
L04739	Calcium ATPase isoform 1	6.6
U14398	Synaptotagmin IV homolog	4.9
U63840	Nucleoporin p54	5.3
X53565	TGN38	3.9
M80550	Adenylyl cyclase	3.9
U12184	Putative G protein coupled receptor	3.7
AF005720	Chloride channel (CIC-2)	3.2
S75687	Glutamate/aspartate transporter	3.1
D63834	Monocarboxylate transporter	3
D64061	Annexin V-binding protein (ABP-7)	3
U50147	Synapse-associated protein 102	3
AF093267	Homer-1b	3
	Intracellular Signaling Proteins	
X56729	Calpastatin	24.7
D88190	Protein kinase (PASK)	16.9
AJ224680	Glutamic-acid rich protein	7.1
S83194	Ca^{2+}/calmodulin-dependent protein kinase IV	6.9
AF084205	Serine/theronine protein kinase (TAO1)	6.2
U77626	Formin binding protein 21	5.9
AF067727	Smad1	3.9
D45920	130kDa-Ins(1,4,5)P3 binding protein	3.7
U26356	S100A1	3.7
AF022085	Guanine nucleotide binding protein beta 4	3.4
M18330	Protein kinase C delta	3.3
U77880	Rolipram-insensitive phosphodiesterase type 7	3.2
D14591	MAP kinase kinase	3
M31809	Calcineurin A-beta	3
	Schwann Cell Proteins	
X06554	MAG	22.4
Z29649	Periaxin	10
K03242	MPZ	6.2
	Transcription Factors	
AF000943	TFIIA large subunit	9.9
L27663	Brn-2	6.3
U67082	KRAB-zinc finger prot. KZF-1	5.2
AB001349	Steroidogenic acute regulatory protein	4.8
AF023087	Nerve growth factor induced factor A (NGFI-A)	4.7
X06769	c-Fos	4.7
L46816	Elongin A	3.2
	Cell Adhesion	
AF020046	Integrin alpha E2	8.6
U20121	Type XI collagen alpha-1	8.3
S72407	Laminin M	4.7
AB004278	Protocadherin 2	3.9
D25290	K-cadherin	3.6
	Immunological Proteins	
Z22812	Interleukin-1 receptor type 2	5.5
D42117	CD30	5.3
M58587	Interleukin 6 receptor ligand binding chain	4.1
Y13972	MHC class I-related protein	3.9
U57062	Natural killer cell protease 4	3.6
D45240	MHC class II	3.1

Accession Number	Gene	Fold Induction
	Cell Metabolism	
AF072865	Thioredoxin reductase	19.4
AF035943	Uncoupling protein-3	13.7
AB011068	Type II iodothyronine deiodinase	10.4
E04239	Choline kinase	5.1
J02585	Stearyl coA destaurase	4.6
M29649	HMG coA reductase	4.5
M26594	Malic enzyme	4.5
S63521	Glucose-regulated protein (GRP78)	4.5
X59608	RNA polymerase II-c	4.2
X12459	Arginosuccinate synthetase	3.9
AB016800	7-dehydrocholesterol reductase	3.5
AB004096	Lanosterol 14-demethylase	3.3
L13722	Geranylgeranyl transferase component A	3.3
AF089039	N-ethylmaleimide sensitive factor	3.1
M83745	Kex2/furin homologue	3.1
M15944	Enkephalinase	3
AF067790	palmitoyl-protein thioesterase (PPT-2)	3
D14046	DNA topoisomerase IIB	3
M55017	Nucleolin	3
	Growth Factors/Receptors	
AF042714	Neurexophilin-4	15.3
M58040	Transferrin receptor	9.9
M77809	Betaglycan	8.9
AF071204	Stem cell factor KL-1 precursor	8.4
M31603	Parathyroid hormone-like peptide (PTHrP)	7.8
L31619	Nicotinic acetylcholine receptor alpha 7 receptor	8.1
S54212	Ciliary neurotrophic factor receptor alpha	7
M32167	Vascular endothelial cell growth factor	5.3
X55246	Glycine receptor alpha-1	4.4
L19341	Activin type I receptor	4.4
X13412	Flk protein	4.4
Z83757	Growth hormone receptor	4.3
AF041082	Robo1	4.3
L20913	Vascular endothelial growth factor form 3	4.2
L00111	Calcitonin	3.8
U08260	N-methyl-D-aspartate receptor NMDAR2D	3.1
Z34004	Growth hormone-releasing hormone	3.1
M38061	Glutamate receptor (GluR-B)	3.1
	Cytoskeletal Proteins	
Z12152	Neurofilament protein middle (NF-M)	3.5
X17682	Microtubule associated protein MAP2c	3.4
	Unknown	
Y09000	Dendrin	18.9
X59864	ASM15	13.1
D25543	Golgi-associated protein GCP360	11.8
AF041107	Tulip 2	4.8
D84418	HMG2	4.3
U32681	Ebnerin mRNA	3.8
AB014722	rSALT-1(806)	3.6
X80395	rVAT	3.2
AF054270	Prolactin-inducible protein (PIP)	3

Genes found to be induced by Egr2 after GeneChip analysis were first grouped functionally and then were sorted by their fold induction. Those genes addressed in the Results and Discussion are underlined.

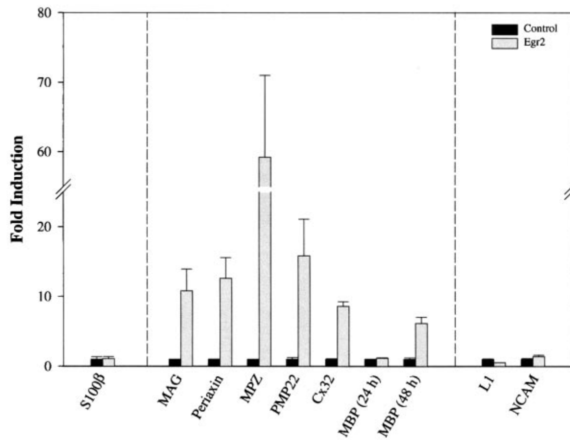


Figure 3. Egr2 Activates Expression of Myelin Genes in Schwann Cells

Quantitative RT-PCR of genes expressed in mouse Schwann cells was performed after infection with control (black bars) or Egr2 expressing adenovirus (gray bars). S100, a pan Schwann cell marker, and nonmyelinating Schwann cell markers, L1 and NCAM, were uninduced, while Schwann cell myelin proteins (MAG, periaxin, MPZ, PMP22, Cx32, and MBP) were induced by Egr2 expression. Note that MBP expression was measured at both 24 hr (MBP [24 hr]) and 48 hr (MBP [48 hr]).

Neuropathy-Associated Egr2 Mutants Act as Dominant Negatives to Inhibit Egr2-Mediated Target Gene Expression in Schwann Cells

Having identified a set of genes that are regulated by Egr2 both in vitro and in vivo in Schwann cells, we first sought to determine whether the EGR2 DBD mutants found in patients with inherited peripheral neuropathies could also regulate the target genes found by expression profiling. To this end, mouse Schwann cells were infected with adenoviruses expressing either Egr2 or the EGR2(S382R, D383Y) mutant, which was identified in a patient with a severe inherited peripheral neuropathy (CHN), and quantitative RT-PCR for several Egr2-responsive essential myelin genes was performed (Figure 5A). Consistent with gel shift experiments and data derived from transfected luciferase reporters, the neuropathy-associated EGR2(S382R, D383Y) mutant was unable to activate MAG, periaxin, PMP22, or MPZ transcription.

Next, we addressed whether these Egr2 DBD mutants would dominantly inhibit wild-type Egr2 induction of target genes identified by microarray expression analysis. Mouse Schwann cells were infected with adenoviruses expressing Egr2 and Egr2(S382R, D383Y) in ratios varying from 1:0.1 to 1:3 and quantitative RT-PCR was performed to monitor induction of target genes from their endogenous chromosomal loci. From this analysis, we found that indeed this mutant dramatically affected the ability of wild-type Egr2 to regulate its target genes, even at a 1:1 ratio. For instance, the activation of all of the myelin genes we examined (MPZ, periaxin, PMP22, and MAG) was significantly inhibited (Figure 6). When we examined L1, which is not responsive to Egr2, no differences in expression were observed in response to the addition of the Egr2 mutant. Conversely, we found that the Egr2-mediated induction of PTHrP expression was dramatically increased in the presence of the Egr2 mutant. To ensure that PTHrP was not induced by expression of mutant Egr2 alone, we monitored PTHrP expression after infection with Egr2(S382R, D383Y)-expressing adenovirus. Consistent with gel shift and transfected luciferase reporter experiments (Warner et al., 1998; Warner et al., 1999), as well as results obtained from monitoring the endogenous expression of essential myelin genes (see Figure 4), no induction of PTHrP expression was observed after expression of the DBD mutant Egr2 protein alone (data not shown). Taken together with the observation that a 50% decrease in MPZ *or* PMP22 expression results in peripheral neuropathy (Adlkofer et al., 1997; Martini et al., 1995b) and that MAG, periaxin, PMP22, and MPZ are dominantly inhibited by DBD mutant Egr2 to levels much below 50% of wild-type levels (5%, 30%, 20%, and 1% respectively), it is clear that the dominant-negative function of mutant Egr2 proteins alone is sufficient to explain the peripheral myelinopathy observed in these patients. Furthermore, the aberrant gene expression resulting from this dominant-negative effect suggests that Egr2 is influenced by adaptor proteins that can serve as either repressors or activators and can be sequestered by Egr2 DBD mutants (see Discussion).

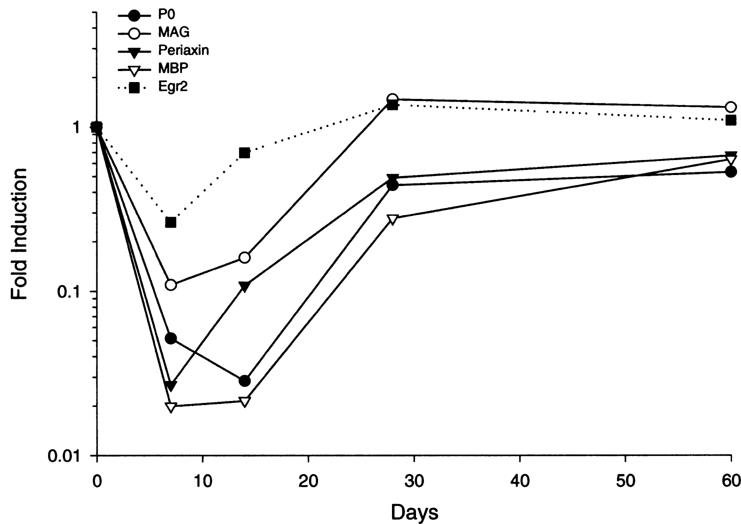
It is somewhat surprising that equal gene dosage of the mutant *Egr2* alleles could so profoundly interfere with the activity of wild-type Egr2. One possibility, which has been reported to account for the dominant-negative function of IκBα, PML-RARA, and Mdm2 mutants, is protein stability. (Kubbutat et al., 1999; Traenckner et al., 1995; Yoshida et al., 1996). To test if the DBD mutants of Egr2 also demonstrate increased stability, QT-6 cells were transfected with wild-type or mutant *Egr2* expression constructs, and Egr2 protein levels were assayed (Figure 5B). Wild-type Egr2 was found at low levels in these cells, whereas all three of the Egr2 DBD mutants accumulated to high levels. Thus, it is possible that this enhanced stability results in an accretion of Egr2(S382R, D383Y) and the other Egr2 DBD mutants to levels that are sufficient to effectively inhibit wild-type EGR2 function in Schwann cells of these patients.

Postnatal *Egr2*-Deficient Schwann Cells Can Express Critical Myelin Genes after Reintroduction of Egr2

To explain the severe impairment of peripheral nerve myelination and decreased expression of several critical myelin genes in *Egr2*-deficient Schwann cells, it has been hypothesized that *Egr2* is required for induction of genes required for myelination or, because *Egr2* is expressed in Schwann cell progenitors as early as E10.5 (Topilko et al., 1997), that lack of Egr2 results in the developmental arrest of Schwann cells at an early stage. Thus, one important question regarding the Schwann cells found in patients harboring *EGR2* mutant alleles is whether they are irreversibly arrested at an early developmental stage or whether they are simply unable to execute the myelination program.

We reasoned that if the defect in *Egr2*-deficient Schwann cells is a block in the myelination program itself, then reintroduction of Egr2 may be sufficient for induction of critical myelin genes. However, if they were arrested at an early developmental stage, then reexpres-

A



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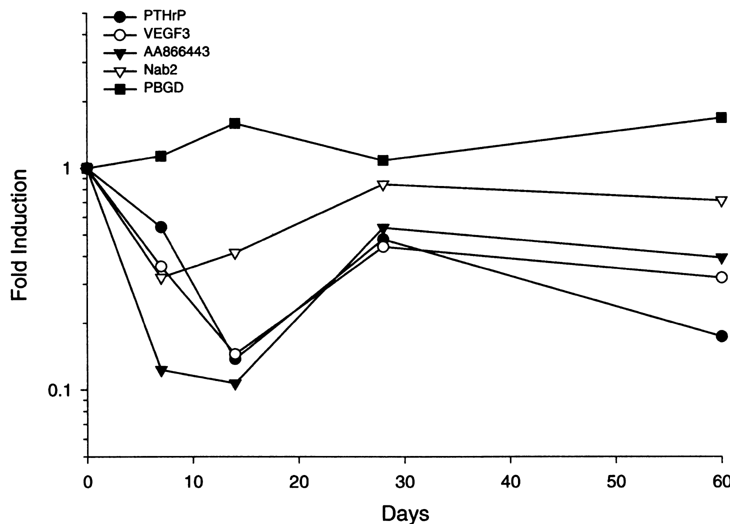

Figure 4. Egr2 Target Genes Are Expressed in Parallel with Egr2 after Nerve Injury

Expression of *Egr2* and the myelin genes (A) and other Egr2 targets (B) were analyzed after nerve injury. Note that reexpression of *Egr2* preceded the other genes. Unlike porphobilinogen deaminase (PBGD), a pseudogene-free reference gene (control), the newly identified targets, PTHrP, VEGF3, AA866443 (an EST with no homology to known genes), Nab2, and the myelin genes (MPZ, MAG, periaxin, and MBP) paralleled the *Egr2* expression kinetics.

sion of Egr2 would not be expected to induce expression of genes characteristic of myelinating Schwann cells (e.g., MPZ, periaxin, and MAG). To examine this question, we infected neonatal Schwann cells from mice expressing extremely low levels of Egr2 that do not effectively myelinate axons ($Egr2^{lo/lo}$) (R. Nagarajan, et al., in preparation) with Egr2-expressing adenovirus and monitored the expression of the newly identified Egr2 target genes. After reexpression of Egr2, we found that the expression of genes required for normal peripheral nerve myelination in the *Egr2* mutant Schwann cells was equivalent to that observed in infected wild-type Schwann cells (Figure 7), indicating that the myelination-permissive environment that is established as Schwann cells mature is present in the Egr2-deficient Schwann cells. Indeed, in cells with a nonpermissive environment, such as epithelial cells or fibroblasts, Egr2 overexpression does not result in myelin gene expression (data not shown). These results indicate that Egr2-deficient Schwann cells are not arrested at an immature state, but, instead, that Egr2 deficiency leads to a failure to execute the myelination program.

Transcriptome Analysis of Cultured Schwann Cells

One extremely powerful feature of global genomic microarray expression analysis is the ability to assess and establish a molecular "fingerprint." Comparison of these unique fingerprints from several induction paradigms or cell types facilitates identification of genes involved in common processes (Hughes et al., 2000). Thus, we performed a detailed analysis of the transcriptome of cultured Schwann cells by surveying the most highly expressed genes from the control sample (Table 2). Because cultured Schwann cells mimic the expression profile of nonmyelinating Schwann cells, we reasoned that this analysis would reveal important genes required for normal function of nonmyelinating Schwann cells. As expected, abundant genes such as cyclophilin and β-actin were included in this list, and several genes

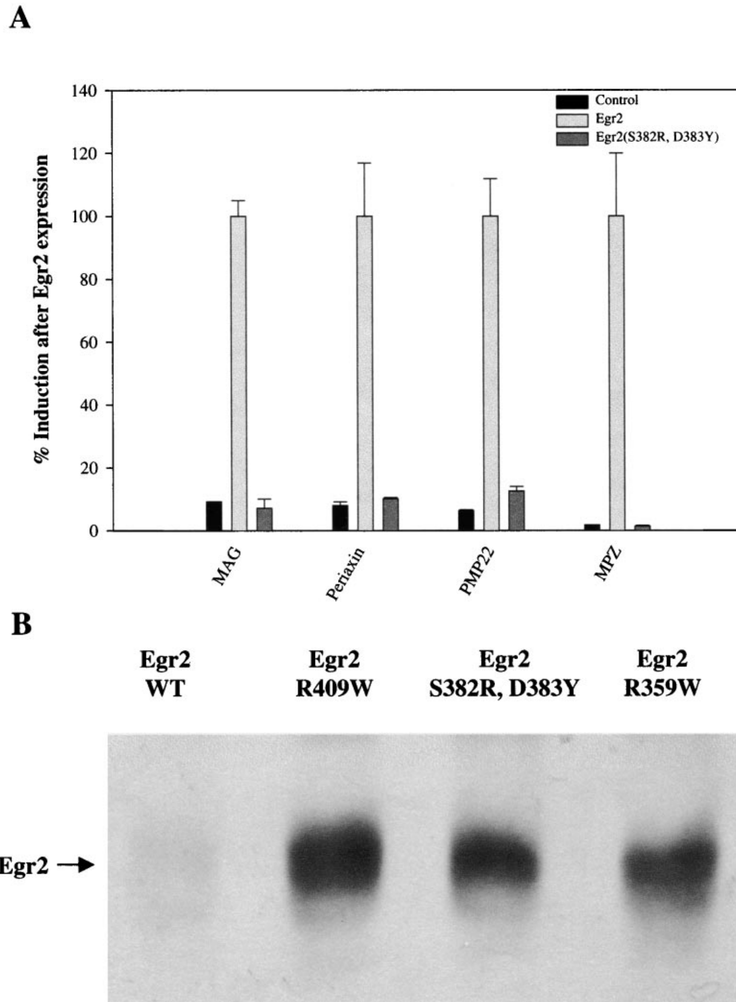

Figure 5. The Neuropathy-Associated Egr2 (S382R, D383Y) Mutant Does Not Activate Expression of Myelin Genes and Accumulates to High Levels in Transfected Cells

(A) Expression of MAG, periaxin, PMP22, and MPZ were analyzed by quantitative RT PCR after infection of mouse Schwann cells with control (black bars), wild-type Egr2 (light gray bars), or Egr2(S382R, D383Y) (dark gray bars). Egr2(S382R, D383Y) was unable to induce expression of any of the myelin genes or of PTHrP (data not shown). Gene induction is represented as percent induction after expression of wild-type Egr2 in Schwann cells.
(B) Individual cultures of QT-6 cells were transfected with expression constructs for wild-type *Egr2* and three mutant *Egr2* alleles found in patients with hereditary peripheral neuropathies. Lysates were made from the cells 48 hr after transfection and examined by Western blotting using a polyclonal antibody directed against Egr2.

known to be expressed in Schwann cells, such as α-1 type I collagen, annexin V, and SPARC (Gillen et al., 1995; Rushton et al., 1999; Spreca et al., 1992) were highly expressed as well. Future analyses aimed at comparing this data set with those derived from astrocytes, oligodendrocytes, and/or myelinating Schwann cells should yield valuable information such as which genes are commonly expressed in all glial cell types versus genes which are uniquely expressed in specific glial cell types or states.

Discussion

Both mutational analysis of patients with peripheral neuropathies and studies of mutant mice have firmly established Egr2 as a critical transcriptional regulator in Schwann cells. However, comparison of the phenotype of the *Egr2* heterozygote mice with the disease presentation of patients who harbor DBD mutations in *EGR2* results in a conundrum. Namely, while *Egr2*$^{+/-}$ mice are phenotypically normal, patients who are heterozygous for a DBD mutant *EGR2* allele are afflicted by one of a spectrum of peripheral myelinopathies (CHN to CMT). In this study, we demonstrate that, although these DBD mutant Egr2 proteins are encoded by loss-of-function alleles, they also dominantly inhibit wild-type Egr2-mediated induction of critical myelin genes, thus elucidating the mechanism by which these mutants cause a severe impairment of myelination in patients. Furthermore, we show that, in addition to genes encoding myelin proteins, Egr2 coordinates expression of multiple genes encoding enzymes required for synthesis of critical myelin lipids. Finally, we show that reexpression of Egr2 in postnatal *Egr2*-deficient Schwann cells is sufficient for induction of genes critical for myelination, implying that the normal function of Schwann cells in patients with inherited peripheral neuropathies attributed to mutations in EGR2 might be restored by reintroduction of EGR2.

Neuropathy-Associated *EGR2* DBD Mutations Inhibit Expression of Genes Encoding Myelin Proteins

While patients carrying mutations in the *EGR2* DBD exhibit peripheral nerve myelinopathies in the heterozygous state, *Egr2* heterozygote mice do not display these deficits. Despite these observations, which have led to the hypothesis that the DBD mutant alleles in these patients encode dominant-negative inhibitors, in vitro gel shift and luciferase reporter assays have not sup-

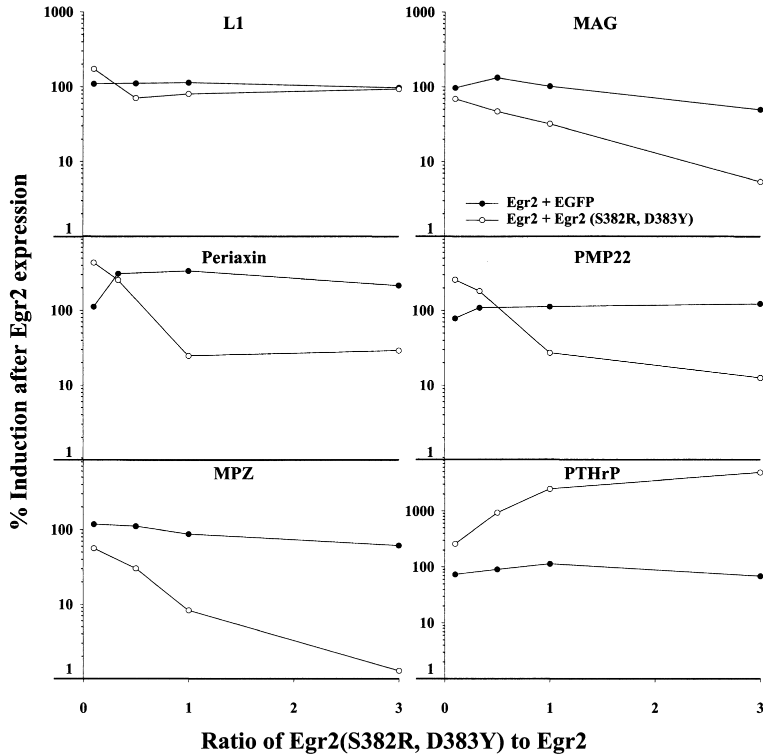

Figure 6. The Neuropathy-Associated Egr2 (S382R, D383Y) Interferes with Wild-Type Egr2-Mediated Gene Activation

Target gene expression was analyzed after co-infection with Egr2 and increasing titers of EGFP (black circles) or Egr2(S382R, D383Y) (white circles) expressing adenoviruses. The ratio of the titer of either EGFP or Egr2(S382R, D383Y) to wild-type Egr2 expressing adenovirus is plotted on the x axis. Expression of L1 (control) (A) was unaffected by Egr2(S382R, D383Y), while expression of MAG (B), periaxin (C), PMP22 (D), and MPZ (E) is decreased and expression of PTHrP (F) is increased.

ported a dominant-negative activity for the Egr2 DBD mutants. However, when we examined mRNA levels of Egr2 target genes (identified in our microarray analysis) from their native chromosomal loci after coexpression of wild-type Egr2 and Egr2(S382R, D383Y), we found that their expression was indeed altered by the mutant protein. Most genes were dramatically repressed (MAG, PMP22, periaxin, and MPZ) by Egr2(S382R, D383Y) coexpression, whereas one gene (PTHrP) was greatly induced. The modulation of wild-type Egr2 activity by these mutant proteins is likely associated with their ability to interact with other proteins, allowing them to sequester Egr2 coactivators or corepressors that are crucial for its function.

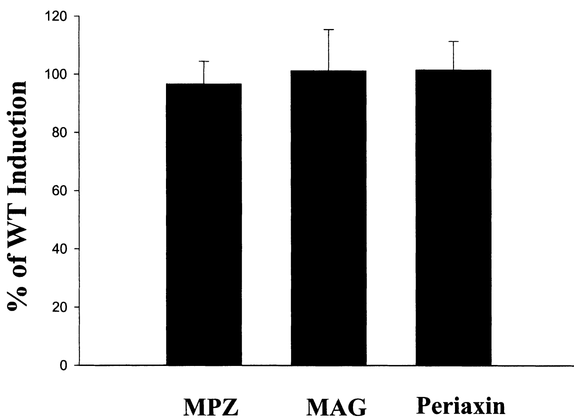

Figure 7. Postnatal *Egr2*-Deficient Schwann Cells Express Genes Critical for Myelination upon Reintroduction of *Egr2*

Schwann cells derived from either wild-type or *Egr2*$^{lo/lo}$ P0 mouse pups were cultured and infected with Egr2-expressing adenovirus for 24 hr. Quantitative RT-PCR of each of the indicated target genes was performed. The fold induction of each target gene after infection with Egr2-expressing adenovirus in *Egr2*$^{lo/lo}$ Schwann cells is expressed as the percent of induction observed in wild-type Schwann cells. Induction of MPZ, MAG, and Periaxin in *Egr2*$^{lo/lo}$ and wild-type Schwann cells is equivalent.

These data, along with the observation that the DBD mutant proteins accumulate to high levels when compared to wild-type Egr2, suggest that patients harboring these *EGR2* mutations have improper peripheral nerve myelination as a result of inappropriate expression of critical myelin proteins. Namely, it is likely that dominant-negative inhibition of MAG, periaxin, PMP22, and MPZ to much less than 50% of wild-type levels is responsible for the inherited neuropathy observed in these patients. In addition, a careful quantification of mutant protein levels in these patients may reveal differences between the alleles and might ultimately provide an explanation for the differing severity of the myelinopathies observed in patients with different *EGR2* DBD mutations.

Egr2 Regulation of Nab2 in Schwann Cells Suggests the Nabs Are Critical for Normal Peripheral Nerve Myelination

While it is unknown which domain(s) of Egr2 are required for binding the coactivators/corepressors described above, it is interesting to note that Nab2, one member of a two gene family (Nab1 and Nab2) of Egr transcriptional modulators, was induced by *Egr2* expression and was coregulated with *Egr2* after nerve injury in Schwann cells. The Nab proteins were initially identified in a yeast two-hybrid screen via their interaction with members of the Egr family (Egr1, Egr2, and Egr3) through the association of two conserved domains, the R1 domain

Table 2. Top 50 Most Highly Expressed Genes in Cultured Schwann Cells

Accession Number	Gene	Expression Level	Accession Number	Gene	Expression Level
L12383	ADP-ribosylation factor 4	30114	X54640	OX47 antigen	22081
M27207	Alpha-1 type I collagen	29451	X15030	Cytochrome c oxidase subunit Va	22062
S79304	Cytochrome oxidase subunit I	29253	L13039	Annexin II	22029
M19533	Cyclophilin	28491	D86641	FK506-binding protein 12	21982
U53184	Estrogen-responsive uterine mRNA	27552	D10706	Ornithine decarboxylase antizyme	21940
D10952	Cytochrome c oxidase subunit Vb	27353	AJ004912	Integral membrane protein Tmp21-I (p23)	21915
J03627	S-100 related protein	26380	D17445	14-3-3 protein eta-subtype	21896
Y13714	Osteonectin	26091	L42855	RNA polymerase II transcription factor SIII p18 subunit	21684
X02610	Non-neuronal enolase	25682	L11007	Cyclin-dependent kinase 4	21633
U95052	Translation repressor NAT1	25431	S82383	Slow-twitch alpha TM/hTMnm homolog	21608
D14688	Myosin regulatory light chain	24772	V01217	Beta-actin	21607
U03390	Protein kinase C receptor	24653	D12770	Mitochondrial adenine nucleotide translocator	21601
D45247	Proteasome subunit RCX	24550	D30739	14-3-3 protein (mitochondrial import stimulation factor [MSF]) L subunit	21470
M91597	RBL-NDP kinase 18kDa subunit (p18)	24052	D30740	14-3-3 protein (mitochondrial import stimulation factor [MSF]) S1 subunit	21447
U75929	SPARC	23712	M86564	Alpha-prothymosin	21395
U75404	Ssecks 322	23460	M54926	Lactate dehydrogenase A	21360
S77858	Nonmuscle myosin alkali light chain	23441	D10874	16 kDa subunit of vacuolar H(+)-ATPases	21339
AJ001929	CBP-50	23269	L26268	Anti-proliferative factor (BTG1)	21263
D13127	Oligomycin sensitivity conferring protein	22948	M23697	Tissue-type plasminogen activator (t-PA)	21234
S45392	Heat shock protein 90	22800	X02904	Glutathione S-transferase P subunit	21186
U44948	Smooth muscle cell LIM protein (SmLIM)	22745	L12380	ADP-ribosylation factor 1	21092
AF083269	p41-Arc	22568	S45663	SC2 (synaptic glycoprotein)	20988
J02962	IgE binding protein	22426	M14050	Immunoglobulin heavy chain binding protein (BiP)	20980
D42137	Annexin V	22410	U50482	Ubiquitin ligase (Nedd4)	20920
M11942	70 kd heat-shock-like protein	22273	M21060	Superoxide dismutase	20854

The top 50 expressed genes in cultured Schwann cells were sorted in decreasing level of expression. The Average Difference parameter, which quantitates hybridization intensity, is used to represent the expression level of each gene.

of Egr proteins and the NCD1 domain of the Nab proteins (Russo et al., 1993; Russo et al., 1995; Svaren et al., 1996). Although initial experiments with the Nab proteins identified them as corepressors of the Egr transcription factors (Svaren et al., 1996; Swirnoff et al., 1998), a recent study has determined that they can also act as coactivators in specific promoter contexts (Sevetson et al., 2000). In addition, both the Nab1 and Nab2 promoters contain multiple Egr binding sites, and Egr proteins have been shown to regulate Nab expression in other tissues (Mechta-Grigoriou et al., 2000). Importantly, the only recessive *EGR2* mutation, *EGR2(I268N)* (Warner et al., 1998), found in patients with CHN lies within the R1 domain and abolishes Nab interaction, suggesting that Nab proteins (or other modulators) are required for Egr2-mediated expression of genes essential for proper myelin formation. The bimodal nature of the Nab proteins (i.e., they can act as either coactivators or corepressors) may be responsible for the differing effects of mutant EGR2 proteins on individual promoters. Thus, the inability of EGR2(I268N) to interact with Nab proteins may result in aberrant Egr2-mediated gene activation and, ultimately, in patients who are homozygous for this mutant allele, in the development of CHN.

Egr2 Regulates Expression of Genes Encoding Myelin Proteins

To test the hypothesis that DBD mutant *EGR2* alleles found in patients with peripheral neuropathies were acting dominantly to inhibit wild-type induction of critical myelin genes, it was first necessary to identify genes which were regulated by Egr2. By performing microarray expression analysis, we discovered that Egr2 coordinates expression of diverse genes involved in multiple processes required for normal Schwann cell function. Foremost on this list of processes is the program of myelin synthesis.

Myelin formation is a complex process that requires synthesis of a variety of lipids, cholesterol, and myelin proteins. The majority of the work in this field has focused on genes encoding myelin proteins. In particular, mutations in *MPZ*, *PMP22*, *Cx32*, and *periaxin* have been found in patients afflicted with peripheral myelinopathies ranging from mild forms of hereditary neuropathy with liability to pressure palsies to the more severe CHN cases (Boerkoel et al., 2001; Guilbot et al., 2001; Lupski et al., 1991; De Jonghe et al., 1997; Raeymaekers et al., 1991). Results from transgenic mice have complemented these studies. Namely, analyses of mice that are deficient for one or more of the six myelin genes—periaxin, MAG, MPZ, PMP22, Cx32, and MBP—have revealed multiple essential roles for these proteins, both in myelin formation and maintenance (Adlkofer et al., 1995; Anzini et al., 1997; Carenini et al., 1997; Carenini et al., 1998; Carenini et al., 1999; Giese et al., 1992; Gillespie et al., 2000; Li et al., 1994; Martini et al., 1995a; Montag et al., 1994; Neuberg et al., 1998). However, the *most severe* peripheral nerve myelination defect is seen in *Egr2*-deficient mice, which have congenitally amyelinated nerves secondary to a uniform arrest of all Schwann cells in the initial stages of axonal wrapping. The severity of the myelination defect, coupled with the fact that Egr2 is a transcriptional regulator, suggests

that Egr2 coordinately regulates a myelination program in Schwann cells. We have demonstrated that this is indeed the case, as *Egr2* expression *alone* is sufficient for activation of periaxin, MAG, MPZ, PMP22, Cx32, and MBP expression in Schwann cells and that dominant-negative inhibition by DBD mutant Egr2 results in an abrogation of this coordinated myelin gene activation program.

Egr2 Regulates Other Genes Involved in Proper Peripheral Myelin Formation

While the genes encoding the myelin proteins have been implicated as possible target genes of Egr2, the potential role of Egr2 in other processes, which are also critical for proper peripheral nerve myelination, has not been described. Our microarray expression analysis has revealed that Egr2 may also coordinate myelin lipid assembly, as it regulates multiple genes encoding enzymes required for lipid and cholesterol synthesis. Furthermore, growth factors and their cognate receptors, which have potential roles in providing trophic and mitogenic support to Schwann cells, are also upregulated by Egr2 expression.

The principal lipid components of myelin include oleic acid, which comprises 35%–45% of all lipid in peripheral myelin (Fressinaud et al., 1986); the phospholipid, sphingomyelin; and cholesterol. Our study demonstrates that genes encoding enzymes required for synthesis of each of these critical myelin lipids is regulated by Egr2. First, choline kinase, which is required for the eventual conversion of ceramide to sphingomyelin, was induced 5.1-fold. Another Egr2-induced gene, which is also developmentally regulated in Schwann cells (Garbay et al., 1998), stearyl CoA desaturase, catalyzes the Δ^9 desaturation of stearyl CoA to form oleic acid. Finally, the synthesis and deposition of cholesterol, a major component of myelin, is also thought to be a highly regulated process in Schwann cells (Fu et al., 1998). Cholesterol is synthesized through a series of complex biochemical reactions including formation of mevalonic acid from 3-hydroxy-3-methylglutaryl CoA via the rate-limiting enzyme of cholesterol synthesis, HMG CoA reductase. This enzyme (4.5-fold) along with two others in the cholesterol synthesis pathway, 7-dehydrocholesterol reductase (3.5-fold) and lanosterol 14-demethylase (3.3-fold), were induced by Egr2 expression in Schwann cells. Thus, several enzymes responsible for formation of critical lipid components of myelin are under coordinated Egr2 regulation.

Several genes regulated by Egr2 in Schwann cells (e.g., those encoding myelin proteins—MPZ, PMP22, Cx32, and recently periaxin [Boerkoel et al., 2001; Guilbot et al., 2001; Lupski et al., 1991; De Jonghe et al., 1997; Raeymaekers et al., 1991]) are mutated in patients with inherited neuropathies. In families with inherited neuropathies in which the mutated gene is unknown, a search for mutations in other Egr2-regulated genes should be performed. For example, a recent study indicated that Fray, a serine-threonine protein kinase, is required for axon ensheathment (Leiserson et al., 2000). In this study, we show that PASK, the mammalian homolog of Fray, is induced 16.9-fold by Egr2 in Schwann cells. Mutations in neurexin IV also result in ensheathment defects (Baumgartner et al., 1996). Interestingly, we have found that neurexophilin-4, which interacts with neurexins, is induced 15.3-fold by Egr2 in Schwann cells. Thus, our data immediately suggest two genes where a search for mutations is likely to be fruitful.

After nerve injury, the expression of *Egr2* and many of the myelination-associated genes decreases dramatically. From our microarray analysis, we identified other Egr2 target genes in Schwann cells that mimic the nerve injury-induced expression pattern of those encoding myelin proteins. Namely, two different splice variants of VEGF as well as one of their receptors (flk-1) were induced by Egr2 expression in Schwann cells. VEGF is known to be a potent mitogen for endothelial cells and is critical for vasculogenesis and angiogenesis (Neufeld et al., 1999). In addition, investigators have found that Schwann cells express flk-1 and that VEGF promotes survival and proliferation of Schwann cells (Sondell et al., 1999). In a recent study, direct application of VEGF intramuscularly either immediately or 10 days after a hypoxic insult greatly alleviated the resulting ischemic peripheral neuropathy (Schratzberger et al., 2000). In fact, the authors suggest that VEGF abrogates the motor and sensory loss by promoting increased neovascularization of the vasa nervorum, nutrient arteries within nerves, and by enhancing Schwann cell survival, proliferation, and migration to repair the damaged area. In light of these studies as well as our findings, it is possible that Egr2 may regulate expression of both VEGF and its receptors during embryonic development to promote vasculogenesis in the developing nerve. Thus, induction of VEGF and flk-1 by Egr2 in Schwann cells could not only promote autocrine survival and proliferative effects, but it might also facilitate angiogenesis through the trophic and mitogenic effects of VEGF on endothelial cells.

Elucidation of Disease Mechanisms Using Neurogenomics

One powerful feature of the global genomic microarray expression technology is the ability to compare data sets derived from experiments which may compare different induction paradigms, cell or tissue types, or mutants. For example, Hughes et al. (2000) conducted microarray expression profiling from 300 different mutations and chemical treatments in *S. cerevisiae* and identified affected cellular pathways by pattern matching. These pattern matching techniques, which include agglomerative hierarchical clustering, k-means clustering, and self-organizing maps, facilitate the sorting of genes into classes and allow the rapid discernment of an unknown gene's function. For example, Hughes et al. identified cellular functions of uncharacterized ORFs simply by the fact that expression profile of these unknown genes coclustered with genes with a known biological function. Thus, the capability to compare multiple data sets derived from diverse expression profiling experiments can dramatically accelerate the determination of gene function, an exciting prospect, particularly in light of the recent completion of the sequence of the human genome.

With respect to the data generated in this study, it will be interesting to compare this data set with those derived from myelinating Schwann cells, Schwann cells

from sciatic nerve injury, and/or Schwann cells from animal models of diabetic and toxin-induced neuropathies. Information from such analyses will be important in deciphering the molecular program elaborated by the Schwann cell to myelinate or to provide trophic support for an axon. Furthermore, such comparisons can be expanded to include other glia such as oligodendrocytes and astrocytes to identify common genes required for normal function of glial cell types or to discover conserved genes required for both central and peripheral nerve myelination.

Examination of oligodendrocytes is particularly relevant with respect to the Egr proteins as Egr1 is known to be expressed in oligodendrocyte progenitors and is induced upon differentiation (Sock et al., 1997). Furthermore, several groups have demonstrated that Egr1 regulates PDGF-A chain (Silverman et al., 1997; Svaren et al., 2000) and that PDGF-A deficient mice have a central nervous system dysmyelinating phenotype due to reduced numbers of oligodendrocytes (Fruttiger et al., 1999). These studies are particularly intriguing in light of recent findings that certain forms of multiple sclerosis are caused by a primary dysfunction in oligodendrocytes (Lucchinetti et al., 2000). Thus, it will be interesting to evaluate the role of the Egr proteins, particularly Egr1, in the pathogenesis of multiple sclerosis subtypes which are caused by oligodendrocyte malfunction.

In conclusion, with the use of global genomic microarray expression profiling, we demonstrate that Egr2 coordinates the peripheral nerve myelination program by inducing genes encoding myelin proteins and enzymes required for myelin lipid assembly. Furthermore, we have revealed that DBD EGR2 mutants cause myelinopathy by dominantly inhibiting wild-type EGR2 induction of these newly identified target genes. Finally, data generated in this study in combination with other expression profiling experiments will be invaluable in unraveling the molecular program required for normal central and peripheral nerve myelination and will ultimately facilitate design and implementation of effective therapeutic treatments for demyelinating diseases such as HMSN and multiple sclerosis.

Experimental Procedures

Cell Culture, Adenoviral Production, and Infection

Rat Schwann cells were purified from P0-P2 sciatic nerves as previously described (Brockes et al., 1979). Briefly, sciatic nerves from P0-P2 rat pups were isolated and treated with collagenase (0.1%) for 1 hr at 37°C. After trituration, dissociated cells were plated on collagen-coated plates. To eliminate fibroblasts, the cells were subjected to three successive rounds (48 hr each) of cytosine arabinoside (10 μM) (Sigma, St. Louis, MO) treatment followed by a 24 hr recovery period in DMEM with 10% FCS. At this point, greater than 99% of remaining cells were Schwann cells. Schwann cells were expanded by adding bovine pituitary extract (20 μg/ml) (Sigma) and forskolin (2 μM) (Sigma). Mouse Schwann cells were isolated from sciatic nerves of P0-P2 pups and were used directly for experiments 5–6 days later.

Adenoviruses expressing EGFP, Egr2, and Egr2(S382R, D383Y) were made essentially as described (Ehrengruber et al., 1998; He et al., 1998). Viral titers were determined by following the Tissue Culture Infectious Dose 50 ($TCID_{50}$) Method from the AdEasy Vector System Application Manual (Version 1.2) published by Qbiogene (Carlsbad, CA). Schwann cells in culture were subjected to adenoviral infection in defined medium (N2) for 24 hr at 37°C (1.9×10^7 pfu/ml) (Bottenstein and Sato, 1979). At this titer and time of adenoviral exposure, greater than 95% of Schwann cells were infected as determined by GFP fluorescence and no cytopathic effects were observed. Furthermore, a time course of expression after infection with Egr2-expressing adenovirus demonstrated that Egr2 mRNA levels were maximal 20 hr after infection. Thus, RNA was harvested 4 hr after the peak of Egr2 expression (i.e., 24 hr after infection) to allow for target gene induction. For cultures where RNA was isolated 48 hr after infection, cultures were washed after 24 hr with PBS followed by reincubation in N2 for an additional 24 hr.

GeneChip Hybridization

Hybridization probes for GeneChip analysis were synthesized as described from poly(A)$^+$ RNA prepared from cultures of Schwann cells that had been infected with either the adenovirus expressing Egr2 or the control adenovirus. The poly(A)$^+$ RNA was converted to double-stranded cDNA using an oligo dT primer containing the T7 promoter, and this was used to prepare biotinylated cRNA using the Bioarray HighYield kit (Enzo, Farmingdale, NY) according to the manufacturer's directions. The biotinylated cRNA probes were fragmented and applied as described (Lipshutz et al., 1999; Lockhart et al., 1996) to Rat Genome U34 GeneChip arrays (Affymetrix, Santa Clara, CA). The signal intensities from hybridized cRNA were quantified, and the GeneChip analysis software was used to identify differentially expressed genes.

Sciatic Nerve Crush

All surgical procedures followed the NIH guidelines for care and use of laboratory animals at Washington University. Male Sprague–Dawley rats (200–300 g) were anesthetized, and the right sciatic nerve was injured at the hip level by compressing the nerve with forceps for 30 s. The contralateral nerve was exposed, but left uninjured (control). After the indicated length of time, the animals were euthanized and decapitated for immediate collection of tissues for RNA isolation.

Cell Lines and Transfection

Culture conditions for the CV-1 cell line have been described previously (Paulsen et al., 1992). CV-1 and rat Schwann transfections were performed in 12-well plates (Corning, Corning, NY) using 3.5×10^4 cells per well. All transfections were performed essentially as described (Russo et al., 1993), using 250 ng of the luciferase reporter, 50 ng of a CMV driven lacZ reporter, and the indicated amounts of the expression plasmids. Bluescript plasmid (Stratagene, La Jolla, CA) was added as required to make a total of 1 μg DNA per transfection. The average luciferase activity of duplicate samples was normalized to the β-galactosidase activity from the transfected lacZ reporter. Means and standard deviations of two separate transfection experiments are shown.

Quantitative RT-PCR (TaqMan) Analysis of Gene Expression

Total RNA was purified and 1 μg of RNA was used to prepare cDNA essentially as described (Lee et al., 1996). Expression levels of genes were measured by quantitative RT-PCR analysis using the TaqMan 7700 Sequence Detection System (Perkin Elmer, Wellesley, MA). Real time detection of PCR product accumulation was monitored using the increase in fluorescence of the SYBR-GREEN dye as described (Morrison et al., 1998). Relative expression levels of these genes in each sample were determined using a standard curve of serial dilutions of cDNA samples containing the highest expression of these genes. Average fold induction relative to control infected cells was determined after normalizing to the amount of GAPDH present in each sample. Standard deviation is obtained from triplicate measurements of each sample. Primer sequences used for quantitative analysis of each gene are available upon request.

Immunoblot Analysis

Quail fibroblast (QT6) cells (2×10^5 cells per 3.5 cm dish) were transfected with 10 μg each of expression constructs for wild-type and mutant versions of Egr2 (Warner et al., 1998). After 48 hr, cells were washed twice with phosphate-buffered saline and then lysed in Laemmli buffer. Lysates were boiled for 10 min, electrophoresed on a sodium dodecyl sulfate 10% polyacrylamide gel, and trans-

ferred to a nitrocellulose membrane (Midwest Scientific, St. Louis, MO). After overnight blocking with Tris-buffered saline containing 5% milk, blots were incubated first with an anti-Egr2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml, and then with a horseradish peroxidase-conjugated antirabbit secondary antibody (Jackson Laboratories, Bar Harbor, ME) at a dilution of 1:10,000. Protein blots were washed five times in TBST and visualized by chemiluminescence detection (Amersham, Piscataway, NJ).

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