A double point mutation in the DNA-binding region of Egr2 switches its function from inhibition to induction of proliferation: A potential contribution to the development of congenital hypomyelinating neuropathy

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Received 17 March 2006; revised 16 May 2006; accepted 5 June 2006

Available online 26 July 2006

Mutations in the DNA-binding domain of EGR2 are associated with severe autosomal dominant forms of peripheral neuropathy. In this study, we show that one such Egr2 mutant (S382R, D383Y), when expressed in Schwann cells in vitro, is not transcriptionally inactive but retains residual wild-type Egr2 functions, including inhibition of transforming growth factor-β-induced Schwann cell death and an ability to induce the cytoskeletal protein periaxin. More importantly, this mutant Egr2 has aberrant effects in Schwann cells, enhancing DNA synthesis both in the presence and absence of the putative axonal mitogen, β-neuregulin 1. This is in stark contrast to wild-type Egr2, which causes withdrawal from the cell cycle. Furthermore, mutant Egr2 upregulates cyclin D1 and reduces levels of the cell cycle inhibitor, p27. These observations add significant new evidence to explain how this mutation leads to congenital hypomyelinating neuropathy in humans.

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Keywords: Schwann cell; CHN; Peripheral neuropathy; Krox-20; Proliferation; Neuregulin; Myelin; Mutation; Adenovirus

Introduction

The early growth response gene 2 (Egr2; also named Krox-20) and the associated proteins Nab1 and 2 are essential transcriptional regulators of Schwann cell myelination (Le et al., 2005a,b; Nagarajan et al., 2001; Topilko et al., 1994). Therefore, it is not surprising that mutations in EGR2 can lead to myelin abnormalities and severe peripheral neuropathies in humans. These include congenital hypomyelinating neuropathy (CHN), Dejerine Sottas syndrome (DSS) and Charcot–Marie–Tooth 1 (CMT1). At present 9 different mutations have been identified in EGR2 (Bellone et al., 1999; Mikesova et al., 2005; Numakura et al., 2003; Pareyson et al., 2000; Timmerman et al., 1999; Vandenberghe et al., 2002; Warner et al., 1998; Yoshihara et al., 2001).

The majority of these mutations occur in the DNA-binding domain and follow a dominant mode of inheritance in affected families. The autosomal dominant S382R, D383Y double point mutation, is associated with the most serious clinical phenotype, identified in a 7-year-old girl diagnosed with CHN. A sural nerve biopsy revealed absence or loss of myelin in virtually all axons and her nerve conduction velocities were severely slowed (under 10 m/s; Warner et al., 1998). The underlying mechanism by which this mutation leads to such severe disruption of the myelination process is of great interest. It is known that this mutant has minimal DNA-binding activity but retains the ability to weakly transactivate an artificial luciferase promoter with Egr2-binding sites (Warner et al., 1999). Furthermore, experiments in mouse Schwann cells in vitro demonstrated that in contrast to wild-type Egr2, this mutant fails to activate transcription of myelin genes, and that it acts as a dominant-negative protein by inhibiting myelin gene expression driven by wild-type Egr2 (Nagarajan et al., 2001). This mechanism would help to explain why the mutation is inherited in a dominant fashion since studies on mouse mutants reveal that one copy of Egr2 is sufficient to sustain normal myelination (Topilko et al., 1994; Zorick et al., 1999).

In the present work, we have examined the functional properties of the S382R/D383Y Egr2 mutant using cultured rat Schwann cells. In agreement with previous work, we find that mutant Egr2 prevents wild-type Egr2 from inducing the expression of the major myelin protein, protein zero (P0). Furthermore, mutant Egr2 fails to induce P0 expression. Nevertheless, we find that mutant Egr2 retains some functions of the wild-type protein. It induces endogenous expression of the myelin-related protein periaxin and Nab2, albeit at lower levels than wild-type Egr2. Moreover, mutant Egr2 is at least as effective as the wild-type protein in preventing Schwann cell death and in common with wild-type Egr2, it suppresses expression of c-Jun. Significantly, we find that mutant...
Egr2 induces aberrant effects when expressed in Schwann cells. Mutant Egr2, under normal culture conditions or in the presence of β-neuregulin-1 (NRG1), induces Schwann cell proliferation, activates the ERK/MAPK kinase pathway and suppresses expression of the cell cycle inhibitor p27. This stands in clear contrast to wild-type Egr2, which inhibits Schwann cell proliferation (Parkinson et al., 2004). These effects are less marked when mutant Egr2 is expressed at higher levels within Schwann cells, when it behaves more like a wild-type allele. These observations add significant new aspects to the functional repertoire of the S382R/D383Y EGR2 mutant protein and will contribute to a comprehensive understanding of why this protein has such devastating consequences when expressed in human Schwann cells.

Materials and methods

Materials

Monoclonal antibodies against phosphorylated ERK1/2 and β-tubulin were purchased from Sigma-Aldrich, Inc. Polyclonal antibodies to cyclin-dependent kinase 2 (CDK2) and p27 and monoclonal antibody to cyclin D1 were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antibody against BrdU was purchased from Roche Diagnostics Ltd. Monoclonal antibody against c-Jun was purchased from BD Biosciences. Monoclonal antibody to Egr2 was bought from Covance, Inc. (Cambridge Biosciences, UK). Polyclonal antibody to GFP was purchased from Molecular Probes. Polyclonal antibody against β-periaxin was a gift from Dr. Peter J. Brophy (Gillespie et al., 1994). Monoclonal antibody to P 0 was a gift from Dr. Juan Archelos (Archelos et al., 1993). Antibody for L1 labeling of rat Schwann cells was prepared from the ASCS4 hybridoma cell line (Sweedner, 1983). Polyclonal antibody (IC4) against NAB2 was a gift from Dr. Judith Johnson (Kirsch et al., 1996). Biotinylated anti-mouse Immunoglobulin (Ig) and streptavidin-Cy3 were purchased from Amersham International plc. Rhodamine-conjugated goat anti-mouse Ig was purchased from MP Biomedicals, llc (Cappel). Cy3-conjugated donkey anti-rabbit Ig was purchased from Jackson Immunoresearch Laboratories, Inc. Goat anti-rabbit and goat anti-mouse horse radish peroxidase-conjugated antibodies were obtained from Promega Inc. Anti-mouse and anti-rabbit Cy5-conjugated secondary antibodies were purchased from Biological Detection Systems, Inc. The MEK inhibitor, U0126 was purchased from Calbiochem, EMD Biosciences, Inc. The JNK inhibitor, SP600125 was purchased from Affiniti Research Products, Ltd. The DJNK1 peptide was a gift from Dr. Husseyin Mehmet (Borsello et al., 2003). Adenoviral constructs expressing GFP, GFP/wild-type Egr2 and GFP/mutant Egr2 (S382R/D383Y) are a gift from Dr. J. Milbrandt (Washington School of Medicine, St. Louis, MO; Nagarajan et al., 2001). Recombinant TGFβ1 and neuregulin β1 (referred to as NRG1) were purchased from R&D Systems, Inc. Sources of other reagents have been detailed elsewhere (Morgan et al., 1991, 1994; Jessen et al., 1994; Dong et al., 1995; Stewart, 1995; Meier et al., 1999; Parkinson et al., 2001, 2003, 2004).

Cell culture

Schwann cells were prepared from the sciatic nerve and brachial plexus from newborn or 3-day-old rats (Brockes et al., 1979; Morgan et al., 1991). Schwann cells for TGFβ-induced apoptosis experiments were prepared by immunopanning and used directly (Dong et al., 1999). For immunocytochemistry experiments, serum-purified, immunopanned or retrovirally infected Schwann cells were plated at a density of 5000 cells in a 15 μl drop on poly-d-lysine/laminin coated coverslips. Cells were then cultured in supplemented medium (Jessen et al., 1994) containing 10^{-6} M insulin, referred to as defined medium (DM), to which was added 0.5% fetal calf serum (FCS; TCS Cellworks, UK) and adeno- natively infected as described previously (Parkinson et al., 2001). For preparation of adenovirally infected cells for Western blot, serum-purified Schwann cells were immunopanned and expanded for 5 days (1 passage) in DMEM supplemented with 3% FCS/2 μM forskolin/10 ng/ml NRG1 and infected as described previously (Parkinson et al., 2001). Western extracts were probed with an antibody to GFP, to ensure equal levels of infection between conditions. For retroviral infection of Schwann cells, cells were purified and expanded essentially as above and infected using retroviral supernatant from GP+E packaging cells as described previously (Parkinson et al., 2001, 2003).

Cell survival assay

Immunopanned Schwann cells from a newborn rat were infected as previously described (Parkinson et al., 2001) with GFP control, GFP/wild-type Egr2 or GFP/mutant Egr2 (S382R, D383Y) adenoviruses, in DM containing 10^{-6} M insulin. Cells were placed into fresh medium after 24 h. After 48 h, cells were then either fixed (time zero control), or treated with or without TGFβ (20 ng/ml) for a further 24 h and then fixed for immunocytochemistry. Results shown are pooled from three individual experiments. Cell survival in this assay was measured by recording the number of GFP-positive Schwann cells at the end of the experiment as a percentage of the number of GFP-positive Schwann cells that had been plated successfully at the beginning of the experiment. Dying cells were identified by Hoechst nuclear labeling, as described previously (Parkinson et al., 2001).

BrdU incorporation assay

Serum-purified Schwann cells were infected as previously described (Parkinson et al., 2001) with GFP control, Egr2 wild- type or mutant Egr2 adenoviruses, in DM containing 10^{-6} M insulin and 0.5% FCS. Cells were, again, placed in fresh medium 24 h after infection and NRG1 (20 ng/ml) was added after a further 22 h. Bromodeoxyuridine (BrdU; 10^{-5} M) was added for the last 20 h of the experiment and cells were fixed at 72 h after infection for analysis by immunocytochemistry. Results shown are pooled from three individual experiments. In order to quantify Schwann cell proliferation counts were performed, recording the number of GFP expressing cells that incorporated BrdU and then converting this to a percentage.

Immunocytochemistry and microscopy

Immunolabeling for BrdU, P 0 and L1 has been described previously (Morgan et al., 1991; Stewart et al., 1993; Parkinson et al., 2003). For all other antibodies, cells were fixed in 4% paraformaldehyde in PBS, pH 7.5, for 10 min. After fixation, cells were permeabilized and blocked in antibody diluting solution (PBS containing 10% donor calf serum, 0.1 M lysine, 0.02% sodium azide) supplemented with 0.2% Triton X-100 for 30 min. Primary antibody to: periaxin was used at 1:8000; c-Jun was used at 1:500;
antibodies to cyclin D1, CDK2 and p27 were used at 1:100 overnight at 4°C. All primary antibodies were diluted in antibody diluting solution (ADS) and 0.2% Triton X-100 except for antibodies to periaxin (ADS only) and NAB2 (used neat). For Egr2/Brdu and periaxin/Brdu double staining the Egr2 and periaxin protocols were done first, including addition of second layer antibodies, and then post-fixed for 5 min in 4% paraformaldehyde, before starting the Brdu protocol. Secondary antibodies conjugated to Cy3, Cy5 or rhodamine in addition to Hoechst nuclear stain (1:1000) were diluted in antibody diluting solution. Coverslips were mounted in citifluor (Citifluor Ltd. London, UK) and examined at room temperature with a Nikon Optiphot-2 fluorescence microscope at ×60 magnification. Confocal microscopy was performed using a Leica DMRE fluorescence microscope with a SP1 confocal head at ×25 magnification and Leica confocal software. For all immunocytochemical experiments, results are pooled from at least 3 individual experiments and an unpaired Student's t-test was performed to determine statistical significance (p<0.05).

Western blot analysis

50 μg of protein extracts was electrophoresed on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Hybond ECL: Amersham Biosciences), blocked with 5% fat-free milk in Tris-buffered saline (TBS) and incubated with primary antibodies in this solution. Antibody to β-tubulin was used at 1:1000; antibodies to Egr2, c-Jun and P0 were used at 1:2500; antibody to periaxin was used at 1:15000; Antibodies to cyclin D1 and p27 were used at 1:500; antibodies to phospho-ERK1/2 and GFP were used at 1:5000. Membranes were washed in TBS and secondary antibody, mouse or rabbit Igs conjugated to horse radish peroxidase was added to this solution (1:2000). Specific protein complexes were revealed using a mix of homemade ECL 1 and 2 chemiluminescent reagents. ECL1 solution was made from 2.5 mM luminol, 0.4 mM coumaric acid, 0.1 M Tris (pH 8.5) in distilled water. ECL2 solution was made using 0.1 M Tris (pH 8.5), 0.02% hydrogen peroxide in distilled water. Both solutions were kept at 4°C in dark conditions and mixed 1:1 just prior to use.

Results

Mutant Egr2 dominant negatively inhibits P0 protein expression induced by wild-type Egr2

Previous experiments show that in mouse Schwann cells mutant Egr2 (S382R, D383Y) can dominant negatively inhibit myelin gene mRNA expression using adenoviral co-infection with a wild-type Egr2 construct (Nagarajan et al., 2001). To confirm these findings at the protein level and in the rat model, we used rat Schwann cells that had been retrovirally infected with wild-type Egr2 and then drug selected. These cells were subsequently infected with either a GFP control adenovirus or an adenovirus expressing GFP and mutant Egr2 for 72 h. In this way, we ensured that the population of Schwann cells examined expressed both wild-type Egr2 and the control or mutant Egr2 constructs. Myelin gene expression was monitored by immunocytochemical labeling for the myelin gene P0. We found, as expected, that mutant Egr2 strongly inhibited the expression of P0 protein (p<0.001), normally induced by wild-type Egr2 (Figs. 1A–E).

Mutant Egr2 retains several functions of the wild-type protein

It has previously been shown by a transfection and electrophoretic mobility shift assay, that the Egr2 (S382R, D383Y) mutant can weakly bind DNA and can activate weak expression of an artificial promoter when ectopically expressed in CV-1 cells (Warner et al., 1999). However, expression of mutant Egr2 for 24 h in mouse Schwann cells failed to induce myelin gene mRNA expression (Nagarajan et al., 2001). We wished to determine more conclusively whether this Egr2 mutant retained properties in common with the wild-type allele in Schwann cells or whether it was, in fact, functionally equivalent to a null allele.

Mutant Egr2 induces periaxin and Nab2 expression

First, we tested whether mutant Egr2 induced expression of any myelin-related genes in rat Schwann cells. Schwann cell cultures were infected with adenoviruses expressing GFP and wild-type Egr2, GFP and mutant Egr2 or GFP alone. Seventy-two hours
later, the cells were assessed for expression of \( P_0 \), periaxin and Nab2 by either Western blot or immunocytochemistry. Mutant Egr2 did not induce \( P_0 \) (Fig. 2A), even when we infected with five times the amount of virus normally used for these experiments (results not shown). In contrast, mutant Egr2 still demonstrated an unambiguous ability to induce periaxin, although periaxin levels were significantly lower than those induced by wild-type Egr2 as shown by Western blot (Fig. 2B) and immunocytochemistry \((p<0.05; \text{Figs. 2C–G})\). Furthermore, we found that mutant Egr2 induced nuclear expression of Nab2 \((25.8\pm3.7\%\)\), though, again, this effect was substantially reduced compared to that of wild-type Egr2 \((99\pm0.7\%, p<0.05; \text{Figs. 2H–M})\).

**Fig. 2. Mutant Egr2 induces periaxin and Nab2 expression.** Schwann cell cultures were infected with GFP control (GFP cont), GFP/wild-type Egr2 (Egr2 WT) or GFP/mutant Egr2 (Egr2 Mut) adenoviruses and assessed for \( P_0 \) expression by immunolabeling (A), periaxin expression by Western blot (B) and immunolabeling (C–G), and Nab2 by immunolabeling (H–M). Scale bar, 20 \( \mu \)m. Note that mutant Egr2 does not induce \( P_0 \) but does induce periaxin and Nab2. However, this induction is clearly less than that seen with wild-type Egr2. Arrowheads in panels L and M signify Schwann cells that express a low level of mutant Egr2 adenovirus and fail to upregulate Nab2. Schwann cell nuclei are labeled with Hoechst nuclear dye (Ho). \( \beta \)-Tubulin was used as a loading control for the Western blot. Error bars on the graphs represent one standard deviation of the mean \((n=3)\).
Mutant Egr2 suppresses c-Jun and protects against TGFβ-induced death

Wild-type Egr2, in addition to inducing myelin genes, also downregulates the expression of molecular components of the Jun-NH2-terminal kinase/c-Jun pathway in cultured Schwann cells (Parkinson et al., 2004). To test whether mutant Egr2 downregulates the expression of c-Jun, we infected cells with GFP control or mutant Egr2 adenoviruses for 72 h. Western blotting showed a substantial c-Jun downregulation in cells expressing mutant Egr2 (Fig. 3A). Reduction of L1 expression, seen in Schwann cells expressing wild-type Egr2 (Parkinson et al., 2003), could also be seen in cells expressing mutant Egr2 although this effect was smaller than that seen with the wild-type protein (data not shown).

Adenoviral expression of wild-type Egr2 prevents Schwann cell death in response to transforming growth factor β (TGFβ), an effect related to the ability of Egr2 to suppress the JNK/c-Jun pathway (Parkinson et al., 2001, 2004). To test whether mutant Egr2 could also block TGFβ-induced Schwann cell death, survival assays were performed on immunopanned Schwann cells prepared from newborn animals. Following infection with GFP control, wild-type Egr2 or mutant viruses, cell survival was measured over a period of 24 h with or without TGFβ. This revealed that, in the presence of TGFβ, Schwann cells expressing mutant Egr2 survived (P<0.01) at least as well as wild-type Egr2 cells (Fig. 3B). Additionally, immunolabeling demonstrated that c-Jun was downregulated in the mutant and wild-type Egr2-expressing cells (results not shown). The medium used in this series of experiments was serum-free. Proliferation was, therefore, essentially absent from all experimental conditions (see below). This was confirmed by measuring BrdU incorporation (not shown; see Discussion).

Mutant Egr2 shows gain-of-function effects that are the converse of those seen with the wild-type protein

Egr2 has a key role in removing Schwann cells from the cell cycle at the onset of myelination. Thus, expression of Egr2 is sufficient to block Schwann cell proliferation in the presence of the axonal mitogen β-neuregulin-1 (NRG1) and in Egr2−/− and Egr2 lo/lo mice Schwann cells continue to proliferate and do not myelinate (Topilko et al., 1994; Parkinson et al., 2004; Le et al., 2005a; Zorick et al., 1999). To test whether mutant Egr2 could also block proliferation in response to NRG1, we infected Schwann cells with GFP control virus, wild-type Egr2 or mutant Egr2 viruses for 72 h. 48 h after infection, some coverslips were treated with NRG1 (20 ng/ml) while others served as controls. BrdU was added for the last 20 h of the experiment. Surprisingly, we found that mutant Egr2, rather than inhibiting proliferation in the presence of NRG1, boosted the level of BrdU incorporation by 29.2±4.79% (P<0.01; Fig. 4A). Furthermore, even in the absence of NRG1, mutant Egr2 raised the levels of Schwann cell BrdU incorporation to 22.5±0.99% (P<0.01; Fig. 4B). Under these normal culture conditions proliferation rates are minimal in control cells and, as expected, wild-type Egr2 fails to promote Schwann cell proliferation (Fig. 4B; Parkinson et al., 2004). The routine culture medium in these experiments included 0.5% fetal calf serum (see Materials and methods). In the complete absence of serum mutant Egr2 was unable to induce DNA synthesis (data not shown).

Because the MEK/ERK pathway has been implicated in Schwann cell proliferation (Kim et al., 1997), we examined the levels of phosphorylated ERK1/2 in cells expressing mutant Egr2. This revealed that in contrast to wild-type Egr2, mutant Egr2 stimulated ERK1/2 phosphorylation (Fig. 4C). In line with this observation, an inhibitor of ERK1/2 phosphorylation, the MEK1/2 inhibitor, U0126, blocked BrdU incorporation stimulated by mutant Egr2 both in the absence and presence of NRG1 (P<0.05 and <0.000005 respectively, Fig. 4D). The Jun-NH2-terminal kinase (JNK) inhibitor, SP600125 also inhibited mutant Egr2 induced BrdU incorporation in the absence and presence of NRG1 (P<0.005 and <0.0005 respectively, Fig. 4D) a result confirmed by using the highly specific JNK blocking peptide, D-JNK1 (Borsello et al., 2003, P<0.05 and <0.0005 respectively, Fig. 4E). These results are consistent with the known involvement of the JNK/c-Jun pathway in Schwann cell proliferation (Parkinson et al., 2004).

In further contrast to the function of the wild-type protein, mutant Egr2 also markedly suppressed levels of the cell cycle inhibitor p27 (Figs. 4F and G). p27 protein is present in quiescent Schwann cells but its expression is elevated by wild-type Egr2, presumably as part of the mechanism through which wild-type Egr2 inhibits cell division (Tikoo et al., 2000; Parkinson et al., 2004).

As expected, given the mitogenic effect of mutant Egr2, we found that mutant Egr2, even in the absence of NRG1, induced

Fig. 3. Mutant Egr2 suppresses c-Jun and cell death. (A) Western blot of c-Jun in Schwann cells infected with GFP control (GFP cont), GFP/wild-type Egr2 (Egr2 WT) or GFP/mutant Egr2 (Egr2 Mut) showing that mutant Egr2 reduces the expression of c-Jun, a protein associated with immature Schwann cells. β-Tubulin was used as a loading control. (B) The graph demonstrates that mutant Egr2 functions similarly to wild-type Egr2 by inhibiting TGFβ-induced Schwann cell death. Immunopanned Schwann cells were infected with GFP control (GFP cont), GFP/wild-type Egr2 (Egr2 WT) and GFP/mutant Egr2 (Egr2 Mut) adenoviruses for 48 h. Some cultures were then fixed (time zero control) or cultured with or without TGFβ (20 ng/ml) for a further 24 h. Values are given for the number of surviving GFP-positive cells as a percentage of the number of GFP-positive Schwann cells at time zero. The error bars represent one standard deviation of the mean (n=3).
nuclear expression of the early cell cycle marker, cyclin D1 ($P<0.001$; Figs. 5A–D). Furthermore, in cells expressing mutant Egr2, the G1-S phase cell cycle enzyme, cyclin-dependent kinase 2 (CDK2) was shifted from the cytoplasm to the nucleus (Figs. 5E and F, I and J). Wild-type Egr2, in contrast, caused a reduction in cdk2 levels (Figs. 5G and H).

At higher concentrations, mutant Egr2 behaves more like wild-type Egr2

Mutant Egr2 binds DNA less strongly than wild-type Egr2. Intriguingly, it demonstrates some normal functions and some aberrant functions (see above). To test whether the aberrant
effects were more prominent at lower Egr2 levels, when DNA binding is likely to be weak, we infected Schwann cells with varying concentrations of mutant Egr2 adenovirus and then measured the amount of periaxin, c-Jun and BrdU incorporation. We found that increasing the level of mutant Egr2 by infecting with up to 5-fold the normal virus quantity increased periaxin induction and reduced c-Jun protein levels still further ($P < 0.001$ and $P < 0.005$ respectively; Fig. 6A). This is likely to be because increasing the mutant Egr2 concentration increases the amount of mutant Egr2 bound to DNA. The level of BrdU incorporation also fell by 20.2±1.78% in Schwann cells infected with 5-fold concentration of mutant Egr2 adenovirus ($P < 0.0005$; Fig. 6B).

To confirm this finding, we double labeled Schwann cells infected with low concentrations of mutant Egr2 adenovirus with antibodies to Egr2 and BrdU. Schwann cells with intense GFP fluorescence, expressed high levels of mutant Egr2 and were BrdU negative, whereas the faintly Egr2- and GFP-positive cells were much more likely to be BrdU-positive (Figs. 6C and D). We also double labeled Schwann cells under the same conditions with antibodies to periaxin and BrdU and found that the cell population that expressed periaxin and the one that incorporated BrdU were non-overlapping (Figs. 6E and F) as expected from previous reports that myelin differentiation and proliferation are incompatible (Morgan et al., 1991).

Fig. 5. Mutant Egr2 regulates cyclin D1 and CDK2. (A) Western blot of cyclin D1 in GFP control (GFP cont), GFP/wild-type Egr2 (Egr2 WT) and GFP/mutant Egr2 (Egr2 Mut) infected Schwann cells confirming that mutant Egr2 can induce the expression of cyclin D1. β-Tubulin was used as a loading control. (B and C) Immunolabeling of Schwann cells, maintained without NRG1, infected with GFP/mutant Egr2 (Egr2 Mut) adenovirus for cyclin D1 demonstrates nuclear staining, a characteristic of proliferating Schwann cells (Kim et al., 2000). Scale bar, 20 μm. (D) The percentage of cyclin D1-positive Schwann cells infected with GFP control (GFP cont), wild-type Egr2 (Egr2 WT) and mutant Egr2 (Egr2 Mut) adenoviruses and maintained without NRG1 ($n=4$). (E–J) Immunolabeling showing cyclin-dependant kinase 2 (CDK2) in Schwann cells infected with GFP control (GFP cont; E and F), GFP/wild-type Egr2 (Egr2 WT; G and H) and GFP/mutant Egr2 (Egr2 Mut; I and J) adenoviruses. Schwann cell nuclei are labeled with Hoechst nuclear dye (Ho). Scale bar, 20 μm. CDK2 is expressed both in the nucleus and the cytoplasm in GFP control cultures and has reduced expression in cells infected with wild-type Egr2. In contrast, cells infected with mutant Egr2 show a distinctly nuclear localization of CDK2 (arrows in I and J), another characteristic of proliferating Schwann cells (Tikoo et al., 2000).
Discussion

We have investigated the functional properties of the S382R/D383Y Egr2 mutant in rat Schwann cells. We find that this mutation does not create an allele that is functionally null, but one that retains a number of functions typical of the wild-type allele. Increasing levels of mutant Egr2 within Schwann cells cause this allele to function more like a wild-type Egr2 molecule, presumably by increasing levels of binding to DNA (Warner et al., 1999). Most importantly for understanding mutant EGR2-related pathology, mutant Egr2 also demonstrates aberrant effects in Schwann cells, namely the induction of proliferation, cyclin D1 and nuclear cdk2 and the suppression of the mitotic inhibitor, p27 that are the converse of the properties of wild-type Egr2. These gain-of-function effects provide additional insight into how this mutation leads to the development of such a severe form of inherited peripheral neuropathy.

Mutant Egr2 can function as a wild-type Egr2 molecule

As mentioned previously, the EGR2 S382R, D383Y mutant was initially identified as a molecule that showed a greatly reduced binding of the Egr2 consensus binding site in an electromobility shift assay (Warner et al., 1999). Further analysis demonstrated that the Egr2 S382R, D383Y mutant could not activate transcription of the myelin-related genes periaxin, myelin associated glycoprotein (MAG), P0 and PMP22, unlike wild-type Egr2 (Nagarajan et al., 2001). Our initial aim was to identify whether the S382R, D383Y mutant retained any Egr2 wild-type functions because it weakly activates a luciferase reporter construct containing Egr2-binding sites (Warner et al., 1999). As expected, we found mutant Egr2 could not induce P0 expression but could still induce low levels of periaxin protein. This finding is in contrast to that of Nagarajan et al. (2001). One possibility for this disparity is that it may reflect a species difference because we used rat Schwann cells for our experiments whereas Nagarajan et al. (2001), used mouse Schwann cells. Furthermore, Nagarajan et al. (2001) measured periaxin mRNA at 24 h after infection of Schwann cells with mutant Egr2 whereas we assayed protein levels after both 48 and 72 h. It is possible that, due to its weakened DNA-binding ability, the induction of periaxin can only be observed at longer time points after adenoviral infection.

Our findings also raise the possibility that there are differences in the Egr2-mediated induction of P0 compared to that of periaxin. The fact that mutant Egr2 never induces P0 expression suggests that a strong Egr2/DNA interaction is required whereas a weaker Egr2/DNA interaction is sufficient to induce periaxin. In a recent study, it was shown that Egr2 directly induces P0 expression in Schwann cells through Egr2-binding sites found in a conserved element within the first intron of the P0 gene (LeBlanc et al., 2006). It is still not known whether Egr2 directly induces periaxin expression in Schwann cells, as Egr2 was unable to activate a reporter construct containing an 8-kb fragment of the periaxin promoter (Parkinson et al., 2003). It should be noted that, it is more correct to think of Egr2 as elevating pre-existing expression of periaxin and P0 in Schwann cells, at the onset of myelination, as both proteins can be detected in Schwann cells before Egr2 is upregulated (Lee et al., 1997; Parkinson et al., 2003). Thus, both genes can be regulated by Egr2-independent mechanisms (Parkinson et al., 2003; Peirano et al., 2000).

An important function of wild-type Egr2 is its ability to promote survival of Schwann cells, even in the presence of death signals such as TGFβ (Parkinson et al., 2001, 2004). This is correlated with the observation that there is increased postnatal Schwann cell death in the Krox-20 null mouse (Zorick et al., 1999). Furthermore, inhibition of the JNK/c-Jun pathway can block Schwann cell death, in vitro and thus the increased survival of Egr2-positive Schwann cells is related to its ability to downregulate c-Jun (Parkinson et al., 2001, 2004). If the S382R, D383Y Egr2 mutant had failed to promote Schwann cell survival,
this might have suggested a further explanation for the severe hypomyelination observed in humans. To our surprise, mutant Egr2 protects newborn Schwann cells from TGFβ-induced death at least as efficiently as wild-type Egr2 and was also able to downregulate expression of c-Jun. The survival experiments were carried out in serum free medium, and therefore, these results were unaffected by cell proliferation (see below). This finding argues against a mechanism involving increased Schwann cell death contributing to the CHN phenotype seen in the patient carrying the S382R, D383Y mutation. In addition, it is remarkable that even though mutant Egr2 has reduced DNA binding it still maintains fully the ability to promote Schwann cell survival. This suggests that the mechanisms that control Egr2-mediated myelin gene expression may differ from those that direct Egr2-mediated cell survival.

**Mutant Egr2 also displays effects that are the converse of wild-type Egr2**

Schwann cells expressing mutant Egr2 demonstrate increased levels of DNA synthesis not only in the presence of the axonally derived mitogen NRG1 but also when NRG1 is absent from the culture medium. Considering the established role of wild-type Egr2 in causing Schwann cell cycle exit, even in the presence of NRG1, this observation was very surprising (Parkinson et al., 2004; Zorick et al., 1999). Nevertheless, it has important consequences considering that a biopsy of the sural nerve of a patient carrying this mutation revealed numerous onion bulbs containing supernumerary Schwann cells, characteristic of increased proliferation of Schwann cells (Warner et al., 1998). Furthermore, evidence from the rat model of CMT1A demonstrates that Schwann cells in demyelinated axonal segments, that presumably give rise to the supernumerary Schwann cells within onion bulbs, express nuclear cyclin D1 and proliferate (Atanasoski et al., 2002), as we observe with mutant Egr2 expressing Schwann cells.

The routine medium for these experiments contained 0.5% calf serum. When this was omitted from the culture medium, we failed to observe significant Schwann cell DNA synthesis. This suggests that the mechanism by which mutant Egr2 stimulates Schwann cell proliferation involves changes in the response to potential mitogens in the cellular environment. We observe that mutant Egr2 can induce nuclear expression of cyclin D1 and cdk2 and reduce levels of the cell cycle inhibitor, p27. Either or both of these responses may contribute to the increased proliferative response of mutant Egr2 expressing Schwann cells. Evidences from cyclin D1-null Schwann cells demonstrate that it is required for Schwann cells to proliferate in culture and after nerve transection (Atanasoski et al., 2001; Kim et al., 2000). Furthermore, overexpression of cyclin D1 in Schwann cells using a retrovirus caused DNA synthesis in a significant proportion of Schwann cells in the absence of growth factor treatment, such as PDGF (Kim et al., 2001). Although reduction in p27 levels is correlated with cell quiescence (Tikoo et al., 2000), it is unknown whether loss of p27 is sufficient to induce Schwann cells to proliferate in culture.

The findings that mutant Egr2 increases ERK1/2 phosphorylation and that the inhibition of the MEK/ERK1/2 and c-Jun/JNK pathways reduces DNA synthesis induced by mutant Egr2, may indicate that mutant Egr2 controls Schwann cell proliferation upstream of the cell cycle regulatory proteins. For instance, mutant Egr2 could increase the expression of growth factor receptors or their activity. We found, however, that the levels of the erbB2 anderbB3 receptor proteins remained unchanged in mutant Egr2 infected cells (not shown). Furthermore, we found that proliferation induced by mutant Egr2 was not inhibited by the use of either of the erbB2 tyrosine kinase inhibitors, AG1478 (Lyons et al., 2005) or GW2974 (not shown). This suggests that mutant Egr2 does not increase erbB2 receptor activity and that it exerts its influence on targets downstream of this receptor. It remains possible that mutant Egr2 alters the expression or phosphorylation state of other growth factor receptors, such as the PDGF receptor. Another possibility arises from the observation that mutant Egr2 induces a significant cell flattening (see Fig. 5 I) suggesting that adhesion between the cells and the substrate may be increased in Schwann cells expressing mutant Egr2. This may be important because Schwann cells were plated onto a laminin substrate and one set of adhesion molecules that may be altered by mutant Egr2 are the integrins, some of which are activated on binding to laminin (Previtali et al., 2001). This is particularly significant given that one of the functions of the integrin receptors is to modulate growth factor signaling (for review, see Guo and Giancotti, 2004).

**Mutant Egr2 demonstrates concentration-dependent effects**

Schwann cells tend to incorporate BrdU when they express lower levels of mutant Egr2 whereas Schwann cells expressing higher levels of mutant Egr2 are less likely to proliferate but instead tend to differentiate and express myelin-related markers such as periaxin. The difference in functional consequence to the Schwann cell, depending on the level of mutant Egr2 expressed, may be linked with either the level of DNA binding of mutant Egr2 or with its ability to associate with other proteins. A recent publication showed that peripheral nerve myelination requires cooperation between Egr2 and its co-factors Nab1 and Nab2 and that Egr2 induces Nab1 and Nab2 as well (Le et al., 2005b). We observe that mutant Egr2 has reduced ability to induce Nab2 and this may contribute to the dysfunction of the Egr2 S382R, D383Y mutant. Interestingly, when Egr2 is expressed in the absence of both Nab1 and Nab2, it not only fails to induce significant levels of myelin genes, such as P0, PMP22 and periaxin but also activates genes that are not activated when Nab1 and Nab2 are present. In addition, the Egr2 I268N mutant, which does not bind Nab proteins and causes recessive CHN, also activates aberrant genes (Le et al., 2005b; Nagarajan et al., 2001).

**Egr2 mutations in inherited peripheral neuropathies**

This is the first study to demonstrate that a mutation in Egr2, which causes dominant CHN, can lead to aberrant function when expressed in Schwann cells. The fact that mutant Egr2 can induce proliferation may help explain the presence of supernumerary Schwann cells in nerves biopsies from patients carrying this mutation (Warner et al., 1998). A previous study showed that the Egr2 S382R, D383Y mutant can dominantly inhibit wild-type Egr2 function (Nagarajan et al., 2001). We confirm that this mutant can act as a dominant-negative molecule, which is particularly important since this mutation follows an autosomal dominant pattern of inheritance (Warner et al., 1998). It remains to be tested whether the other dominant EGR2 DNA-binding mutations can also act in a dominant negative way. However, it is becoming increasingly clear that these mutations may induce subtly different molecular and cellular phenotypes since they lead to peripheral neuropathies of varying severity. In the case of EGR2 DNA-binding mutants, it...
has already been shown that the increasing ability to bind DNA correlates with the increasing severity of peripheral neuropathy that they cause (Warner et al., 1999). In view of the complex spectrum of normal and abnormal functions shown by the Egr2 S382R, D383Y mutant, it will be important for future studies to investigate the functional properties of other EGR2 DNA-binding mutants in order elucidate the particular mechanisms by which these mutations lead to peripheral nerve dysfunction.

Acknowledgments

We would like to thank Jeffrey Milbrandt, Peter Brophy, Julian Archelos, Judith Johnson, Hussein Mehmet and David Becker for the gift of reagents. We also thank Daniel Cianister for assistance with the confocal microscopy.

This work was supported by a Wellcome Trust Program grant to K.R. Jessen, R. Mirsksy and D.B. Parkinson and a M.R.C. studentship to P. Arthur-Farraj.

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Acknowledgments

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Schwann cells and regulation of cyclin D1 expression in an animal


