Functional consequences of mutations in the early growth response 2 gene (EGR2) correlate with severity of human myelinopathies

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The early growth response 2 gene (EGR2) is a Cys₂His₂ zinc finger transcription factor which is thought to play a role in the regulation of peripheral nervous system myelination. This idea is based partly on the phenotype of homozygous Krox20 (Egr2) knockout mice, which display hypomyelination of the PNS and a block of Schwann cells at an early stage of differentiation. Mutations in the human EGR2 gene have recently been associated with the inherited peripheral neuropathies Charcot–Marie–Tooth type 1, Dejerine–Sottas syndrome and congenital hypomyelinating neuropathy. Three of the four EGR2 mutations are dominant and occur within the zinc finger DNA-binding domain. The fourth mutation is recessive and affects the inhibitory domain (R1) that binds the NAB transcriptional co-repressors. A combination of DNA-binding assays and transcriptional analysis was used to determine the functional consequences of these mutations. The zinc finger mutations affect DNA binding and the amount of residual binding directly correlates with disease severity. The R1 domain mutation prevents interaction of EGR2 with the NAB co-repressors and thereby increases transcriptional activity. These data provide insight into the possible disease mechanisms underlying EGR2 mutations and the reason for varying severity and differences in inheritance patterns.

INTRODUCTION

The early growth response 2 gene [EGR2 (1), also known as Krox20 (2)] is a member of a family of early growth response (EGR) genes which includes EGR1 (3) [also known as NGFIA (4), Krox24 (5) and Zif268 (6)], EGR3 (7) and EGR4 (NGFIC) (8). The EGR proteins contain a zinc finger domain homologous to the one identified in the Drosophila melanogaster Krüppel (Kr) gap gene (9,10). The similarity with Kr suggested that the products of these genes were DNA-binding proteins involved in genetic control at the transcriptional level. Experimental evidence has shown that the EGR genes encode transcription factors which contain Cys₂His₂ zinc fingers which bind a GC-rich consensus binding site (11). The EGR proteins mediate a variety of signaling pathways that are important for cellular growth and differentiation and responses to hormonal stimuli (12–16).

EGR2 was initially identified using low stringency hybridization with an Egr1 cDNA probe on a cDNA library constructed from RNA extracted from cells after serum stimulation (1). Extensive nucleotide similarity identified Krox20 as the mouse ortholog of EGR2 (17). Analysis of mouse knockouts has demonstrated that Krox20 is important for hindbrain segmentation and development (13,14) and endochondral bone formation (18,19). In addition, peripheral nervous system (PNS) myelination is absent and Schwann cells are blocked at an early stage of differentiation with reduction in major components of compacted myelin such as P₀ and myelin basic protein, encoded by late myelin genes (15). Krox20 expression is specifically associated with the onset of myelination in the PNS and is essential for the final differentiation of myelinating Schwann cells (20). These data suggest that Krox20 and its human homolog EGR2 are transcription factors required for the transactivation of PNS myelination-specific genes.

In support of the role of EGR2 in PNS myelination, several mutations have recently been identified in patients with one of the following inherited peripheral neuropathies: Charcot–Marie–Tooth type 1 (CMT1), Dejerine–Sottas syndrome (DSS) or congenital hypomyelinating neuropathy (CHN) (21,22; Fig. 1). This group of disorders represents a spectrum of related clinical phenotypes, ranging from the least severe CMT1 phenotype, which results in a slowly progressive distal muscle atrophy and decreased motor nerve conduction velocities (NCVs) with onset in late childhood or adulthood (23), to DSS, in which similar features are exhibited with increased severity (i.e. slower NCVs and earlier age of onset) (24), and finally to CHN, which like Krox20 homozygous knockout mice have hypomyelination of the PNS (25). Point mutations in several known myelin genes, PMP22, MPZ and Cx32 (26),
as well as a 1.5 Mb tandem duplication at 17p11.2–p12 which alters dosage of PMP22 (27,28), have previously been associated with these disorders. The association of different mutations within the same gene with various clinical phenotypes is a common finding in this group of peripheral neuropathies and further supports the contention that these disorders represent a spectrum of related phenotypes due to an underlying defect in PNS myelination (29).

Understanding the functional consequences of these \( \text{EGR2} \) mutations is important for the elucidation of the molecular mechanisms underlying the associated neuropathies. The position of the various mutations in known functional domains of \( \text{EGR2} \) gives insight into the potential effect these mutations may have on protein function. The I268N mutation affects the R1 domain that interacts with NAB proteins (Fig. 1). The NAB proteins, NAB1 (30) and NAB2 (31), are co-repressors which can repress transcriptional activity of the EGR proteins. This repression requires interaction of the NAB co-repressors (32) with the conserved R1 domain (33) of the EGR transcription factors. A point mutation (I293F) within the \( \text{Egr1} \) R1 domain has been shown to abolish interaction with the NAB proteins, thereby resulting in an increase in transcriptional activity (33). Ile268 in \( \text{EGR2} \) is analogous to Ile293 in \( \text{Egr1} \). Therefore, the I268N mutation in \( \text{EGR2} \) may similarly affect \( \text{EGR2} \) activity.

The location of the three other \( \text{EGR2} \) mutations (R359W, S382R/D383Y and R409W) within the zinc finger domain suggests that these mutations may affect DNA binding (Fig. 1). In different members of the EGR family, both arginines mutated in the neuropathy patients have been shown to be important for direct DNA binding (34–36) and the aspartic acid mutated in the S382R/D383Y mutation has been shown to support the DNA binding interactions (35).

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<th>Mutations</th>
<th>DNA Binding</th>
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<td>R359W</td>
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\( \text{EGR2} \) in peripheral nerve myelin development and maintenance.

**RESULTS**

**Isolation and sequencing of an EST containing the entire coding region of \( \text{EGR2} \)**

Using the published \( \text{EGR2} \) sequence, we identified EST 362693 (GenBank accession no. aa018140) which matched both the 5’- and 3’-ends of \( \text{EGR2} \). Direct sequencing of this clone confirmed the potential sequencing error (presence of only one G instead of two at nucleotide position 1682) in the original \( \text{EGR2} \) sequence (data not shown; GenBank accession no. AF139463) (37). The presence of only one G was confirmed by the loss of an \( \text{StuI} \) restriction site in PCR fragments from several patients and controls (data not shown). A nucleotide polymorphism (A638→T, Pro100→Pro) and an additional sequencing error (A1176→G, M280→V) were also identified (data not shown). The change to Val280 results in an exact match at this position with the mouse Krox20 amino acid sequence. Incorporation of these DNA sequencing changes extends the predicted protein by an additional 20 amino acids at the C-terminus (37). In addition, when the \( \text{EGR2} \) sequence is aligned with the revised \( \text{Krox20} \) sequence, this alignment starts further upstream than identified previously and changes the start methionine to the most 5’ ATG. These modifications result in a 475 amino acid, 51 kDa protein and increase the amino acid identity between Krox20 and \( \text{EGR2} \) from 75 to 89% overall and from 89 to 100% in the zinc finger domain.

**Mutants in the zinc finger domain of \( \text{EGR2} \) inhibit DNA binding**

DNA binding was assayed by electrophoretic mobility shift assays (EMSA) using the consensus \( \text{EGR2} \) binding site (5’-GGCGTGGGGCG-3’) as a probe (11). Protein derived from each mutant construct (I268N, R359W, S382R/D383Y, S382R, D383Y and R409W) was tested for its ability to bind DNA as compared with the wild-type construct. In addition, protein from a nonsense mutation (R405X) that truncates the third zinc finger and C-terminal sequences was used as a negative control. The I268N mutation in the R1 domain showed wild-type
levels of DNA binding, whereas the R359W, R409W and R405X mutant constructs were unable to bind the consensus site (Fig. 2a). The S382R/D383Y mutant showed a severely decreased level of DNA binding as compared with the wild-type (Fig. 2a). Testing for a potential cumulative effect of the S382R/D383Y mutant was evaluated by comparing the signals of the individual mutations (S382R and D383Y) with that of the double mutant. The S382R mutation alone binds as well as the wild-type and the D383Y mutation alone binds at the same low level as the double mutant (Fig. 2a). Therefore, the effect of the double mutant is not cumulative, but only the D383Y mutation affects DNA binding and, thus, it is probably responsible for the phenotype.

Specificity for the EGR2 binding site was demonstrated by a competition assay. The signal observed with probe binding disappears in the presence of excess unlabeled EGR2 consensus binding site competitor as compared with the wild-type (Fig. 2a). Testing for a potential cumulative effect of the S382R/D383Y mutant was evaluated by comparing the signals of the individual mutations (S382R and D383Y) with that of the double mutant. The S382R mutation alone binds as well as the wild-type and the D383Y mutation alone binds at the same low level as the double mutant (Fig. 2a). Therefore, the effect of the double mutant is not cumulative, but only the D383Y mutation affects DNA binding and, thus, it is probably responsible for the phenotype.

Specificity for the EGR2 binding site was demonstrated by a competition assay. The signal observed with probe binding disappears in the presence of excess unlabeled EGR2 consensus binding site competitor, but not with an excess of a non-specific competitor (ERE binding site; 38) (Fig. 2b). The differences in the abilities of the wild-type, I268N and S382R/D383Y to bind to the EGR2 consensus site were examined using a competition assay in which increasing molar amounts of unlabeled EGR2 competitor (0, 10, 30, 100 and 300×) were added to the binding assay (Fig. 3a) and bound probe was quantitated using a phosphorimager (Fig. 3b). This analysis indicates that the wild-type and I268N proteins bind the consensus site with equal affinity, whereas binding by the S382R/D383Y mutant is significantly reduced (Fig. 3b). The differences in the abilities of the wild-type, I268N and S382R/D383Y to bind to the EGR2 consensus site were examined using a competition assay in which increasing molar amounts of unlabeled EGR2 competitor (0, 10, 30, 100 and 300×) were added to the binding assay (Fig. 3a) and bound probe was quantitated using a phosphorimager (Fig. 3b). This analysis indicates that the wild-type and I268N proteins bind the consensus site with equal affinity, whereas binding by the S382R/D383Y mutant is significantly reduced (Fig. 3b).

**EGR2 mutants affect transcriptional activation**

To further investigate the consequences of the mutations on EGR2 function, we performed a series of experiments to test the ability of the mutants to activate transcription of a luciferase reporter construct that is fused to a promoter containing two EGR binding sites. A titration experiment was used to determine the ability of each mutant protein to activate transcription (Fig. 4). We found that both the R409W and R405X mutants totally lacked the ability to activate transcription from the EGR reporter, consistent with their inability to bind DNA (Fig. 4a). The S382R/D383Y mutant activated transcription at a low level. Similar to our observations using the DNA-binding assay, the reduced activity was solely due to the D383Y
mutation (Fig. 4b). The R359W mutant, which showed no binding in the EMSA assay, was able to activate transcription at a very low level (Fig. 4a), suggesting that it retained some low level of DNA-binding activity not detectable by the less sensitive EMSA binding assay.

In addition, the transfection experiments showed a considerable increase in transcriptional activity in the I268N mutant as compared with the wild-type. This was presumably due to the inability of the mutant to bind and be repressed by the endogenous NAB proteins present in the CV-1 cell line. This effect was further demonstrated by titrating increasing amounts of NAB2 expression plasmid in the presence of a constant amount of I268N or wild-type construct. Measuring the fold activation of the I268N mutant compared with the wild-type clearly showed the abrogation of NAB repression on the I268N mutant (Fig. 5).

DISCUSSION

DNA binding and transcriptional activation experiments, using wild-type and myelinopathy-causing EGR2 mutations, provide insights into how mutations in EGR2 may cause disease and result in differences in clinical severity and inheritance patterns. The I268N mutation, located in the R1 domain, probably results in the deregulation of EGR2 and causes CHN through a gene dosage mechanism, much in the same way increased dosage of PMP22 by the CMT1A duplication results in CMT1 (39,40). The recessive nature of this mutation may reflect a threshold effect in which a certain increased protein level of the downstream PNS target gene(s) must be achieved for manifestation of a phenotype.

The other three mutations (R359W, S382R/D383Y and R409W), which occur within the zinc finger domain, affect DNA binding and thereby potentially influence the transcriptional activity of a gene(s) important for PNS myelination. The variation in clinical severity (CMT1 < DSS < CHN) seen with the zinc finger mutations appears to correlate with the level of residual DNA binding. The most severe phenotype (CHN) is associated with the mutant (S382R/D383Y) which confers the greatest level of DNA binding (Fig. 2a) and transcriptional activation (Fig. 4a), while the least severe phenotype (CMT1) is observed in the mutant (R409W) which shows no binding (Fig. 2a) or transcriptional activation (Fig. 4a). These observations suggest an allelic series with an increasing functional defect (R409W < R359W < S382R/D383Y) resulting from differences in binding affinity.

The dominant nature of these human mutations is interesting given that heterozygous Krox20 knockout mice appeared phenotypically normal (13,14). This suggests that rather than acting as loss-of-function alleles, the zinc finger mutations...
may instead be acting as dominant-negative or gain-of-function alleles. In support of a dominant-negative disease mechanism, dominant mutations of these same conserved amino acids in the zinc fingers of the WT1 gene have been associated with Denys–Drash syndrome (DDS) (41) and are known to abolish the DNA-binding ability of the WT1 protein (42). The DDS mutations are proposed to act as dominant-negative alleles because patients with a deletion of the WT1 gene have a less severe phenotype (43) and transcriptional activation by wild-type WT1 has been shown to be inhibited by co-expression of DDS mutant WT1 protein (44). In our transfection assays, we have not observed interference with the activity of wild-type EGR2 when the DNA-binding domain mutants are co-expressed (data not shown). However, our transfection system does not incorporate effects from other factors because the reporter plasmid contains only two binding sites for EGR2. Therefore, the promoter context and abundance of cofactors in our transfection assays probably differs significantly from that of the relevant target promoters in Schwann cells. These zinc finger mutations could also behave as gain-of-function alleles by forming inappropriate interactions with cofactors or by binding to inappropriate targets. Elucidation of the exact pathomechanism underlying these zinc finger mutations will require the identification of the relevant target genes in Schwann cells.

The PNS specificity of the phenotype seen with EGR2 mutations is intriguing since homozygous Krox20 knockout mice manifest findings in a number of different systems: hindbrain segmentation (13,14), bone formation (18,19) and PNS myelination (15). Limitation of the phenotype to the PNS is supported by the lack of any central nervous system (CNS) neurological findings in all four patients and normal MR1 findings in two siblings from the family with the I268N mutation (21,22). Krox20 is known to regulate transcription of HoxB2 (45) and Hox-1.4 (46), genes required for segmentation of the CNS, which is consistent with the observed effect on the hindbrain in Krox20 homozygous knockout mice, but the downstream PNS-specific EGR2 target genes are not known. P0 is a possible downstream target found on the basis of the absence of P0 expression in homozygous Krox20 knockout mice (15). However, Brown and Lenke (47) detected no Krox20 binding to the MPZ promoter by DNase I footprint analysis. Nevertheless, they did show that transfected Krox20 was capable of a 2-fold transactivation of a cloned MPZ promoter linked to a luciferase reporter gene (48). These data demonstrate that Krox20 is capable of transactivating the MPZ promoter, but do not determine whether it does so directly or indirectly. Sequence similarity searches for the EGR2 consensus binding site in the known promoters of PMP22 and Cx32, the other two myelin-specific genes known to be involved in this group of peripheral neuropathies (24), revealed no potential EGR2-binding sites. The specificity of the phenotype may reflect tissue-specific interactions among constituents of the transcriptional machinery, complementation by the different members of the EGR family in the other involved systems and/or sensitivity of the PNS myelin to changes in gene dosage. The PNS myelin has been shown previously to be extremely sensitive to changes in gene dosage: heterozygous duplication/deletion of PMP22 and loss-of-function MPZ alleles result in a peripheral neuropathy phenotype (24).

Our experiments, which determined the functional consequences of EGR2 mutations, indicate that peripheral myelinopathy severity correlates directly with the ability to bind a cis-acting regulatory site in vitro. However, this correlation is based on a limited number of mutations. A complete understanding of the pathway from mutation to specific neuropathy phenotype will require the characterization of additional disease-associated mutations, the identification of downstream PNS-specific target genes of EGR2 and an understanding of the additional factors involved in EGR2 transcriptional regulation. Nevertheless, our findings clearly indicate a prominent role for EGR2 in PNS myelin development and maintenance.

**MATERIALS AND METHODS**

**Plasmids**

All expression constructs were made with the pcDNA3.1(+) vector from Invitrogen (Carlsbad, CA). Wild-type EGR2 coding sequence was PCR amplified from EST 362693 (GenBank accession no. aa018140) using the primers BamHI (5’-CGC-GGATCCCTACCGTATGCCGAGCCCTGAG-3’) and XbaI (5’-GTCTTAGATCAAGGTGTCCGGTGTCGAG-3’). The wild-type PCR fragment was digested with BamHI and XbaI and cloned into the pcDNA3.1(+) vector. Using the wild-type construct as a template, the mutant constructs I268N, S382R/D383Y, S382R, D383Y and R409W were made by sequential PCR steps with mutant primers derived from the genomic sequence and vector primers T7 (5’-TAATACGACTCTATAGGG-3’) and BGH (5’-TAAAGGGACGTCGAG-3’), as described (49). The full-length mutant fragments generated were then cloned into a TA vector (Invitrogen). The mutant EGR2 coding sequences were amplified from these mutant constructs using the BamHI and XbaI primers, digested with BamHI and XbaI and cloned into the pcDNA3.1(+) vector. The R405X construct was generated by a PCR artifact (C1551→T) during the construction of the R409W mutant. The R359W mutant construct was made using the QuiChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The DNA sequences of all constructs were confirmed by fluorescent automated sequencing. The appropriate protein size of products from each construct was shown by analyzing 35S-labeled in vitro coupled transcription and translation synthesized protein made using the TNT system (Promega, Madison, WI) on an SDS–PAGE gel (5% stacking gel and 10% separating gel). The luciferase reporter construct containing the two EGR2 consensus binding sites (8), the CMV-driven lacZ reporter (31) and NAB2 expression vector (31) have been described.

**EMSAs**

The double-stranded EGR2 consensus binding site or ERE probe was made by annealing equal amounts of two complementary oligonucleotides (ERE sense, 5’-GGGAGTCACTTCGTTGGG-GCTTGTAGTCGTT-3’; ERE antisense, 5’-CAGTCAAGACCCCACAATGTCACCTGCAGAGCC-3’; ERE sense, 5’-GTCAAGT-GGTTGTAGTCGTTGACTATGCCTA-3’; ERE antisense, 5’-TTCAGTCTAGTCAGTGGACTATTCATA-3’). Fifty nanograms of the EGR2 consensus binding site probe was end-labeled with 40 µCi [α-32P]dCTP using 2 U DNA polymerase
Klenow fragment. The labeled probe was purified using Sephadex NICK columns from Pharmacia Biotech (Piscataway, NY). DNA binding reactions were performed in a buffer that has been described previously (11), with the addition of 10 pmol single-stranded DNA. Proteins used in all gel shift experiments were synthesized by in vitro coupled transcription and translation using the TNT system (Promega). Two microliters of in vitro translated protein from each expression construct was added to the DNA-binding buffer plus 13 fmol of probe for a total reaction volume of 30 μl and incubated for 30 min at 30°C. Probe bound to protein was separated from free probe as described previously (11). For the competition assays displayed in Figures 2b and 3a, different molar concentrations of unlabelled non-specific competitor (ERE) or specific competitor (EGR2 consensus) were mixed with the DNA-binding reactions before separation on a non-denaturing polyacrylamide gel. For the quantitative analysis shown in Figure 3b, bound probe was quantitated with a phosphorimager and the percentage of bound probe seen with each construct at each competitor concentration was calculated as the ratio of bound probe plus competitor to bound probe without competitor.

Transfections
African green monkey CV-1 cells were cultured as described previously (50). Cells were plated in 12-well plates (Corning, Boston, MA) at 3.5 × 10⁵ cells/ml and grown for 1 day before performing transfections. All transfections were performed essentially as described (33), using 250 ng of the luciferase protein from each expression construct was added to the DNA-binding buffer plus 13 fmol of probe for a total reaction volume of 30 μl and incubated for 30 min at 30°C. Probe bound to protein was separated from free probe as described previously (11). For the competition assays displayed in Figures 2b and 3a, different molar concentrations of unlabelled non-specific competitor (ERE) or specific competitor (EGR2 consensus) were mixed with the DNA-binding reactions before separation on a non-denaturing polyacrylamide gel. For the quantitative analysis shown in Figure 3b, bound probe was quantitated with a phosphorimager and the percentage of bound probe seen with each construct at each competitor concentration was calculated as the ratio of bound probe plus competitor to bound probe without competitor.

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