RESEARCH ARTICLE

Retinopathy Mutations in the bZIP Protein NRL
Alter Phosphorylation and Transcriptional Activity

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The transcription factor neural retina leucine zipper (NRL) is required for rod photoreceptor differentiation during mammalian retinal development. NRL interacts with CRX, NR2E3, and other transcription factors and synergistically regulates the activity of photoreceptor-specific genes. Mutations in the human NRL gene are associated with retinal degenerative diseases. Here we report functional analyses of 17 amino acid variations and/or mutations of NRL. We show that 13 of these lead to changes in NRL phosphorylation. Six mutations at residues p.T50 (c.148T>A, c.148T>C, and c.149C>T) and p.P51 (c.151C>A, c.151C>T, and c.152C>T), identified in patients with autosomal dominant retinitis pigmentosa, result in a major NRL isoform that exhibits reduced phosphorylation but enhanced activation of the rhodopsin promoter. The truncated NRL mutant proteins—p.T50fs (c.148_149insC) and p.P51fs (c.151_152fs)—do not localize to the nucleus because of the absence of bZIP domain. The p.L160P (c.479T>C), p.L160fs, and p.R218fs (c.654delC) mutant proteins do not bind to the NRL-response element, as revealed by electrophoretic mobility shift assays. These three and p.S225N (c.674G>A) mutant show reduced transcriptional activity and may contribute to recessive disease. The p.P67S (c.199C>T) and p.L235F (c.703C>T) variations in NRL do not appear to directly cause retinitis pigmentosa, while p.G122E (c.365G>A), p.H125Q (c.375C>G), and p.L160fs (c.477dup)—do not localize to the nucleus because of the absence of bZIP domain. The p.L160P (c.479T>C), p.L160fs, and p.R218fs (c.654delC) mutant proteins do not bind to the NRL-response element, as revealed by electrophoretic mobility shift assays. These three and p.S225N (c.674G>A) mutant show reduced transcriptional activity and may contribute to recessive disease. The p.P67S (c.199C>T) and p.L235F (c.703C>T) variations in NRL do not appear to directly cause retinitis pigmentosa, while p.G122E (c.365G>A), p.H125Q (c.375C>G), and p.L160fs (c.477dup)—all of which are of uncertain significance. Our results support the notion that gain-of-function mutations in the NRL gene cause autosomal dominant retinitis pigmentosa while loss-of-function NRL mutations lead to autosomal recessive retinitis pigmentosa. We propose that differential phosphorylation of NRL fine-tunes its transcriptional regulatory activity, leading to a more precise control of gene expression. Hum Mutat 28(6), 589–598, 2007. Published 2007 Wiley-Liss, Inc.

KEY WORDS: gene regulation; retinal degeneration; photoreceptor; transcription factor; phosphorylation; gene expression; DNA binding; rhodopsin; neural retina leucine zipper; NRL; retinal development

INTRODUCTION

Retinitis pigmentosa (RP; MIM# 268000) constitutes a group of progressive degenerative diseases of the retina. With a combined incidence of 1:3,500, RP is the most common inherited retinopathy, which is associated with night-blindness, gradual reduction of peripheral visual fields, and eventually loss of central vision [Heckenlively, 1988; Kennan et al., 2005]. To date, 181 retinopathy loci have been mapped and 129 genes identified [Rivolta et al., 2002] (RetNet website; www.sph.uth.tmc.edu/Retnet). In general, RP-associated genes encode components of the phototransduction cascade, retinoid metabolism, photoreceptor structural proteins, intracellular transport proteins, splice factors, and transcription factors [Bessant et al., 2001; Kennan et al., 2005; Rattner et al., 1999]. Neural retina leucine zipper (NRL; MIM# 162080, cone-rod homeobox (CRX; MIM# 602225), nuclear receptor subfamily 2 group E member 3 (NR2E3; MIM# 604485), and Tubbly-like protein 1 (TULP1; MIM# 602280) are among the known and/or putative transcription factors, mutations in which cause RP [Banerjee et al., 1998; Bessant et al., 1999; Boggon et al., 1999; Hagstrom et al., 1998; Haider et al., 2000; Nishiguchi et al., 2004; Sohocki et al., 1998].

NRL, a bZIP transcription factor of the Maf-subfamily, is a phosphorylated protein that is specifically expressed in rod photoreceptors and pineal gland, but not in cones or other cell types [Akimoto et al., 2006; Swain et al., 2001]. Deletion of Nrl (Nrl<sup>−/−</sup>) in mouse results in the retina with no rods but a

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concomitant increase of cone photoreceptors derived from cells fated to be rods [Akimoto et al., 2006; Mears et al., 2001]. NRL is shown to bind to a cis-regulatory sequence (Nrl response element, NRE) in the rhodopsin promoter and enhance its activity in cultured cells alone or together with CRX, NR2E3, and other proteins [Chen et al., 1997; Cheng et al., 2004; Lerner et al., 2001; Mitton et al., 2000, 2003; Pirtler et al., 2004; Rehemtulla et al., 1996]. A number of downstream target genes of NRL have been identified by combining microarray analysis with chromatin immunoprecipitation studies [Yoshida et al., 2004; Yu et al., 2004]. These findings suggest an essential role of NRL in normal rod photoreceptor differentiation and functional maintenance.

A mutation in codon 50 (Serine to Threonine) of NRL was first associated with autosomal dominant RP (ADRP), and this change was suggested to be a hypermorph [Bessant et al., 1999]. NRL mutation screens have now revealed 14 missense and three frameshift sequence variations in patients with retinal disease (Fig. 1; Table 1) [Acar et al., 2003; Bessant et al., 1999; DeAngelis et al., 2002; Martinez-Gimeno et al., 2001; Nishiguchi et al., 2004; Wright et al., 2004; Ziviello et al., 2005]; the mutations associated with ADRP include p.S50L (c.149C>T), p.S50P (c.149T>C), p.S50T (c.149T>A), p.P51S (c.151C>T), and p.P51T (c.151C>A). The putative recessive NRL mutations (p.L75fs [c.224_225insC], p.L160P [c.479T>C]) have been associated with clumped pigmentary retinal degeneration [Nishiguchi et al., 2004]. Other sequence changes are suggested to be of uncertain significance (p.E63K [c.187G>A], p.A76V [c.227C>T], p.D129E [c.387G>A], p.H125Q [c.375C>T], p.P51S, and p.P51T) or considered nonpathogenic (p.P67S [c.199C>T], p.H125Q [c.375C>G], and p.L235F [c.703C>T]) based on inheritance data or clinical findings [Nishiguchi et al., 2004; Ziviello et al., 2005]. Due to a very low occurrence of sequence changes in the NRL gene [Acar et al., 2003], it is possible that some sequence variations predispose certain individuals to photoreceptor disease.

We initiated this study for a comprehensive biochemical evaluation of all detected sequence changes in NRL, with a goal to obtain insights into their contribution to human retinal disease and to permit better understanding of structure–function relationships. Previously, we had described the effect of p.S50T, p.P51S, and p.L160P mutations in NRL on rhodopsin promoter activity in cultured cells [Bessant et al., 1999; Nishiguchi et al., 2004]. Here, we have determined the effects of all 17 reported sequence changes on NRLs phosphorylation, nuclear localization, DNA-binding ability, and transcriptional activation. Our studies demonstrate the role of phosphorylation and validate a critical function of p.S50 and p.P51 residues in modulating NRL's transcriptional activity. Our data also suggest possible contributions of sequence changes in NRL on the pathogenesis of retinal disease.

### MATERIALS AND METHODS

#### Cell Culture and Transfection

COS-1 and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN), at 80% confluency, with plasmid DNA, as previously described [Nishiguchi et al., 2004].

#### Plasmid Construction and Mutagenesis

The wild-type (WT) human NRL cDNA (GenBank #NM_006177.2) was subcloned at the EcoRI–NotI sites in

### Table 1. Summary of the Effects of Mutations/Variations on NRL Function

<table>
<thead>
<tr>
<th>Proband diagnosis</th>
<th>Nucleotide change*</th>
<th>Amino acid change*</th>
<th>Alleles</th>
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<th>NRL isoforms</th>
<th>Subcellular localization</th>
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</tr>
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<tbody>
<tr>
<td>ADRP</td>
<td>c.149 C&gt;T</td>
<td>p.S50L</td>
<td>p.S50L/+</td>
<td>b</td>
<td>6</td>
<td>Nuclear</td>
<td>+</td>
<td>Up</td>
</tr>
<tr>
<td>ADRP</td>
<td>c.151 C&gt;T</td>
<td>p.P51T</td>
<td>p.P51T/+</td>
<td>b</td>
<td>1</td>
<td>Nuclear</td>
<td>+</td>
<td>Up</td>
</tr>
<tr>
<td>CPRD</td>
<td>c.224_255insC</td>
<td>p.L75fs</td>
<td>p.L75fs/p.L160P</td>
<td>b,g</td>
<td>Not detected</td>
<td>cytoplasmic</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>CPRD</td>
<td>c.654 delC</td>
<td>p.R218fs</td>
<td>p.R218fs/+</td>
<td>f</td>
<td>4</td>
<td>Nuclear</td>
<td>+</td>
<td>Down</td>
</tr>
</tbody>
</table>

*The mutation nomenclature uses the first nucleotide of the ATG codon in the cDNA (NM_006177.2) as +1, and the first amino acid of the protein as +1 (NM_006177.2).

**DeAngelis et al. (2002).**

**Bessant et al. (1999).**

**Martinez-Gimeno et al. (2001).**

**Ziviello et al. (2005).**

**Nishiguchi et al. (2004).**

**Wright et al. (2004).**

ADRP, autosomal dominant RP; ADRR, autosomal recessive RP; CDS, cone dysfunction syndrome; CPRD, clumped pigmentary retinal dystrophy; LCA, Leber congenital amaurosis; Mut, mutation; RP, retinitis pigmentosa; Unc, uncertain significance; Var, variation.
the pcDNA4 His/Max C vector (Invitrogen, Carlsbad, CA). The QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used, as described [Nishiguchi et al., 2004], to generate all mutants from the NRL expression construct. All constructs were sequence-verified before use.

**Immunoblot Analysis**

Transfected COS-1 whole cell extracts were solubilized in 2 x SDS sample buffer by heating to 100°C for 5 min and separated by 15% SDS-PAGE. Proteins were transferred to nitrocellulose by electrot blotting, and immunoblot analysis was performed using a mouse monoclonal anti-Xpress antibody (Invitrogen) according to standard protocols [Ausubel et al., 1989].

**32P Metabolic Labeling and Immunoprecipitation (IP)**

Transfected COS-1 cells were metabolically labeled using 0.5 μCi/ml [γ-32P]ATP (GE Healthcare, Piscataway, NJ) as described [Ausubel et al., 1989]. After 1 hr, labeled cells were harvested in PBS containing 1 x protease inhibitors, and sonicated. After cell extracts were preabsorbed with Protein-G agarose beads (Invitrogen), the cell extracts were incubated with anti-Xpress antibody and Protein-G agarose beads overnight at 4°C with gentle shaking. The beads were washed with PBS containing 1% Triton X-100. The proteins were suspended in 2 x SDS sample buffer and then analyzed by SDS-PAGE.

**Phosphatase Treatment**

Transfected COS-1 cells were washed with phosphate buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 x complete protease inhibitor (Roche Applied Science), and treated for 1 hr at 30°C with 80 units of λ-phosphatase (New England Biolabs, Beverly, MA). The reaction was terminated by heating to 100°C for 5 min in 5 x SDS sample buffer, and the samples were subjected to SDS-PAGE.

**Immunocytochemistry**

Transfected COS-1 cells were washed with PBS, fixed using 4% paraformaldehyde/PBS for 10 min, and washed again in PBS. Cells were permeabilized using 0.05% Triton X-100/PBS for 10 min. After washing, a 5% bovine serum albumin (BSA)/PBS solution was applied and the cells were blocked for 30 min. The cells were incubated at 1 hr with an anti-Xpress antibody (1:400 dilution) in 1% BSA/PBS, and with a secondary anti-mouse immunoglobulin G (IgG) Alexa fluor 488 (Molecular Probes, Eugene, OR) (1:400 dilution). Nuclei were counterstained with bisbenzimide, and cells were examined by fluorescent microscopy.

**Electrophoretic Mobility Shift Assays (EMSA)**

Gel shift assays were performed essentially as described [Rehemtulla et al., 1996], with minor modifications. Nuclear extracts from transfected COS-1 cells were prepared using a commercial kit (Active motif, Carlsbad, CA), and expression of mutant NRL protein was normalized by immunoblot analysis. Nuclear extracts were preincubated for 30 min on ice in binding buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol (DTT), 10% glycerol, 2.5 μg/ml poly(dI-dC). Radiolabeled DNA probes containing the rhodopin promoter driving a luciferase cDNA sequence (pBR130-luc), and expression constructs carrying the CRX cDNA (pcDNA4-CRX) and/or NR2E3 cDNA (pcDNA4-NR2E3), as described [Bessant et al., 1999; Nishiguchi et al., 2004], with minor modifications. Increasing amounts (0.01, 0.03, and 0.09, 0.3 μg) of a NRL expression construct containing either WT or NRL mutant/variant was also cotransfected with pBR130-luc (0.3 μg per well), and pcDNA4-CRX and/or pcDNA4-NR2E3 (0.5 μg per well), as indicated for individual experiments. Empty pcDNA4 expression vector and cytomegalovirus-β-gal (0.1 μg per well) were included to normalize for the amount of transfected DNA and transfection efficiency, respectively. The reactions were repeated in triplicate at least three times except for earlier published mutants (p.S50T, p.P51S, p.L160P) [Bessant et al., 1999; Nishiguchi et al., 2004].

**RESULTS**

**Evolutionary Conservation of NRL Variants Identified in Retinopathy Patients**

Evolutionary conservation of amino acid residues can provide significant insights into NRL function. NRL orthologs have been identified in many vertebrates with the exception of chicken [Coolen et al., 2005; Whitaker and Knox, 2004]. To date, 17 mutations and/or variations in the NRL gene have been detected [Bessant et al., 1999; DeAngelis et al., 2002; Martinez-Gimeno et al., 2001; Nishiguchi et al., 2004; Wright et al., 2004; Zivielo et al., 2005]; these include 14 missense and three frameshift mutations (Fig. 1A). All changes have been identified in 12 amino acids; three of these (p.S50, p.P51, p.L160) show more than one alteration. Five (p.S50, p.P51, p.A76, p.L160, and p.R218) of the 12 residues are conserved in all known orthologs of NRL from human to fugu (Fig. 1B). Residues p.P67 and p.L75 are conserved in all orthologs, except zebrafish and frog, respectively (Fig. 1B).

**Effect of NRL Mutations/Variants on Protein Stability and Phosphorylation Status**

In a previous study, NRL isoforms from human retina extract showed a pattern similar to that of transfected COS-1 cells [Swain et al., 2001] or HEK293 cells (data not shown), suggesting that modifications of NRL are congruous among retina and these cell types. We therefore expressed WT and mutant NRL proteins in COS-1 cells to examine their effect on NRL stability and phosphorylation status. In contrast with at least six 35–35-kDa isoforms (including a 4-kDa Xpress epitope) of WT-NRL, all p.S50 and p.P51 mutants showed significant reduction of isoforms, with the appearance of a major 30-kDa band (Fig. 2A). The p.P67S, p.H125Q, and p.S225N proteins displayed patterns equivalent to that of WT-NRL, suggesting that these changes do not affect protein stability or phosphorylation (Fig. 2A). Mutants p.E63K, p.A76V, p.G122E, and p.L160P contained a different isofrom pattern. The p.E63K band sizes were in the WT range, while that of p.L160P were of higher molecular mass. The p.A76V and p.G122E mutants showed significant reduction of isoforms, with alteration. Five (p.S50, p.P51, p.A76, p.L160, and p.R218) of the 12 residues are conserved in all known orthologs of NRL from human to fugu (Fig. 1B). Residues p.P67 and p.L75 are conserved in all orthologs, except zebrafish and frog, respectively (Fig. 1B).
FIGURE 1. A schematic of the human NRL protein, and amino acid sequence alignment of NRL orthologs. A: Arrows indicate altered NRL amino acid residues identified in individuals with retinopathies. MTD, minimal transactivation domain; Hinge, hinge domain; EHD, extended homology domain; BD, basic domain; Leu. Zipper, leucine zipper (NM_006177.2). B: The amino acid sequence of human NRL is aligned with those of chimp, rhesus, cow, dog, mouse, rat, frog, zebrafish, and fugu using ClustalW. Amino acid residues conserved in all orthologs are indicated by an asterisk and reduced identity is shown using either a colon or a dot. Residues with human changes described in the text are shown by arrows.

FIGURE 2. Isoform and phosphorylation analysis of WT and mutant NRL proteins. A: Immunoblot analysis of COS-1 whole cell extracts expressing WT or mutant NRL constructs. NRL protein isoforms were detected using an anti-Xpress antibody. Composite image from multiple immunoblots. B: Metabolic labeling of NRL with 32P. WT, p.S50T, and p.P51S NRL transfected COS-1 cells were radiolabeled with 32P. After solubilization, the NRL proteins were immunoprecipitated using anti-Xpress antibody. C: Alkaline phosphatase treatment of NRL. COS-1 whole cell extracts expressing WT, p.S50T, or p.P51S NRL were treated with or without phosphatase (PPase) and detected with the anti-Xpress antibody.
perhaps due to lower levels or unstable protein (data not shown). WT and mutant NRL constructs were transfected into human Y79 retinoblastoma cells as well. However, transfected NRL isoforms (carrying Xpress tag) could not be detected by immunoblot analysis because of low transfection efficiency (data not shown).

To directly test NRL phosphorylation, we performed metabolic labeling using [γ-32P]ATP and immunoprecipitation using anti-Xpress antibody. WT, p.S50T and p.P51S mutants were phosphorylated, with the mutant proteins showing only the lower isoform(s) (Fig. 2B). Phosphatase treatment of the WT-NRL-transfected COS-1 cell extracts demonstrated a reduction in NRL isoforms, while the treated mutant proteins migrated slightly below the untreated (Fig. 2C). This is consistent with previous studies showing a reduction in NRL isoforms upon phosphatase treatment of human and bovine retina extracts [Swain et al., 2001].

Effect of NRL Mutations/Variants on Nuclear Localization

We then examined the subcellular distribution of mutant NRL proteins in COS-1 cells. All except two of the NRL mutant proteins (p.L75fs, p.L160fs) localized to the nucleus (Fig. 3). Both of these mutations would be predicted to lose their bZIP domain and mislocalized to the cytoplasm. The p.L75fs mutant was essentially undetectable at exposure times equivalent to the other samples (Fig. 3). At higher exposure, p.L75fs had very weak expression in a pattern similar to p.L160fs (data not shown).

Effect of NRL Mutations/Variants on DNA Binding

We previously showed that NRL is bound to NRE in the rhodopsin promoter (rhodopsin-NRE) [Rehemtulla et al., 1996]. We confirmed that COS-1–transfected NRL protein could also bind to the rhodopsin-NRE (Fig. 4A). The intensity of the shifted bands was dramatically decreased by unlabeled rhodopsin-NRE in a concentration dependent manner; however, no change in intensity was detected with the nonspecific (NS) control oligonucleotide, and in fact the NS probe reduced the NS oligonucleotide shifts (Fig. 4A). Subsequent EMSA experiments were performed to investigate whether mutant NRL protein(s) affect rhodopsin-NRE binding. All variations except for p.L160P,
p.L160fs and p.R218fs bound to the rhodopsin-NRE (Fig. 4B). The p.A76V alteration appeared to have lower than WT binding.

**Effect of NRL Mutations/Variants on Transactivation of Rhodopsin Promoter**

We tested the effect of mutations in NRL on their ability to transactivate luciferase reporter activity driven by the bovine rhodopsin promoter in the presence of CRX, as described [Rehemtulla et al., 1996]. All p.S50 and p.P51 mutants showed a statistically significant increase (analysis of variance [ANOVA] with a post hoc test; P < 0.05) in transactivating the rhodopsin promoter when compared to WT-NRL at three of the four DNA concentrations tested (Fig. 5A). The p.P67S, p.A76V, and p.G122E alterations had no change from WT, while p.H125Q gave inconsistent results being significantly higher than WT using 0.03 or 0.09 μg DNA and lower with 0.3 μg NRE DNA (Fig. 5B). Mutations exhibiting lower than WT transactivation were: p.E63K, p.L160P, p.L160fs, p.R218fs, and p.S225N (P < 0.05, in at least three of four DNA concentrations tested; Fig. 5C and D). The p.L235F was significantly lower than WT at only two DNA concentrations (0.01 and 0.3 μg; Fig. 5C).

We then examined whether mutant NRL proteins demonstrate altered transactivation of the rhodopsin promoter in the presence of NR2E3, which also acts as coactivator of rod genes with NRL and/or CRX [Cheng et al., 2004]. The p.S50T exhibited enhanced activation of the rhodopsin promoter when cotransfected with NR2E3 and/or CRX (Fig. 6A and B). The p.P67V and p.A76V did not show significant differences from WT in both experiments, whereas p.G122E and p.H125Q showed higher activities than WT when both NR2E3 and CRX were present (P < 0.05, in at least three of four DNA concentrations tested; Fig. 6B). The p.S50T and p.P51S mutants activated the rhodopsin promoter at higher levels than WT in the absence of CRX and NR2E3 and did not affect NRL's interaction with CRX or NR2E3, as revealed by co-IP experiments (data not shown).

**DISCUSSION**

Diseases associated with mutations in transcription factors can result from altered protein expression, stability, or function. Transient modulation of protein function can occur through posttranslational modification of proteins, such as phosphorylation, acetylation, ubiquitination, and/or sumoylation [Freiman and Tjian, 2003; Yang, 2005]. NRL has several predicted phosphorylation sites (18 serine, nine threonine, and seven tyrosine residues) and two sumoylation sites (Lys-20 and -24; ψ-K-X-E-, where ψ is a hydrophobic residue) [Falquet et al., 2002; Puntervoll et al., 2003]. The six currently known p.S50 and p.P51 NRL mutations lead to ADRP in patients [Bessant et al., 1999; DeAngelis et al., 2002; Martinez-Gimeno et al., 2001; Nishiguchi et al., 2004]. All of these changes consistently display increased NRL-mediated transactivation of the rhodopsin promoter and a loss of NRL isoforms that is consistent with decreased NRL phosphorylation. Hence, NRL's activity appears to be altered by phosphorylation status. The p.S50 and p.P51 residues are located within NRLs minimal transactivation domain (MTD), which is important for the interaction of NRL with TATA-binding protein (TBP) [Friedman et al., 2004]. We hypothesize that phosphorylation status affects NRL's ability to bind TBP and other components of the general transcription machinery. Consistent with this, two serine residues (Ser-14 and -65) in the transactivation domain of the L-Maf protein (Ser-65 corresponds with Ser-50 in NRL), are shown to be important phosphorylation sites for extracellular-signal regulated kinase in vitro, and the p.S14A and p.S65A mutant L-Maf showed higher levels of reporter activity than WT protein [Ochi et al., 2003]. It appears that higher molecular mass isoforms of NRL have additional phosphorylated residues and exhibit lesser transcriptional activation of the rhodopsin promoter. Interestingly, NRL isoforms of higher molecular mass are detected in early developmental stages of mouse retina [Swain et al., 2001]. These isoforms may interact with as yet undiscovered proteins and regulate the expression of different target genes.
In mature rods, higher NRL transcription activity (and less phosphorylated isoforms) would probably be required to maintain high level expression of rod phototransduction genes, such as rhodopsin. To further investigate the role of phosphorylation, we generated NRL mutants with individual and plural serine and threonine residues (Ser-6, 38, 41, 45, 46, 50, 54, 91, and 117, Thr-42 and 52) changed to alanine, and examined their effects on protein isoforms using immunoblot analysis. Almost all of these mutants show reduced number of isoforms, but are not like the p.S50 mutant with a single major protein isoform (A.K., J.S.F., A.S., unpublished data). Even after phosphatase treatment of the wild type protein, more than one NRL band is detected, suggesting additional posttranslational modifications. We propose that the phosphorylation at Ser-50 residue, probably modulated by Pro-51, plays an important role in triggering additional modifications in NRL.

Four other alterations (p.E63K, p.P67S, p.L75fs, and p.A76V) exist in or near NRL’s MTD. The categorization of p.E63K and p.A76V is difficult. p.E63K’s lower transactivation and p.A76V’s decreased binding in EMSAs are consistent with the suggestion that these changes are recessive in nature. The p.E63K variation significantly changes amino acid polarity, which might have an effect on NRL-TBP or other protein–protein interactions, possibly causing reduced transcriptional function of NRL. The p.A76V, however, also activated the rhodopsin promoter at close to WT levels both in the presence and absence of CRX (this study; data not shown). Other studies have also shown transcription factor mutations having differential effects on DNA binding and transactivation [Chen et al., 2002; Lines et al., 2002]. It is possible...
that the substitution of alanine with valine at position 76 affects NRLs conformation, which, in turn, differentially alters DNA-binding and transactivation. Our data shows p.P67S with similar transactivation as WT, indicating that it may not be a clinically-relevant variation. The p.L75fs mutation does not produce a stable, mature protein and has been previously suggested as disease causing when present with another loss of function NRL allele [Nishiguchi et al., 2004].

Within NRLs hinge region, the p.G122E alteration was suggested to be a change of uncertain significance, while p.H125Q was considered a variation [Martinez-Gimeno et al., 2001]. Our current findings do not support the hypothesis that the p.G122E alteration is disease-causing. The p.G122E and p.H125Q alterations were not transcriptionally different from WT when tested with CRX and had an inconsistent pattern; nevertheless, these two showed high activity with the rhodopsin promoter when combined with both CRX and NR2E3. Although these alterations had measurable differences from WT in our assays, we still consider these particular changes to be of uncertain significance. Notably, both p.G122E and p.H125Q exist in the dog and frog orthologs of NRL, respectively. Additional studies are needed to clarify their importance. Maf’s hinge domain is believed to modify the conformation of its N-terminal region and affect accessibility to general transcription factors [Yoshida and Yasuda, 2002]. It is possible that NRL’s hinge region also behaves in a similar manner.

The remaining alterations are observed within the basic leucine zipper domain. The p.L160P and p.L160F are near the DNA-binding basic domain and adjacent to NRLs ancillary DNA-binding region [Kerppola and Curran, 1994]. Both mutations have a remarkably negative impact on NRL function. We suggest that these variants function as recessive alleles and affect the recognition of NRL’s DNA binding site. Furthermore, it is interesting that the p.L160P mutant isoforms migrated more slowly than WT though only a leucine to proline substitution is present. It is possible that this mutation introduces an as yet unknown posttranslational modification, which slows its migration in SDS-PAGE. Reduced DNA binding and transactivation by p.R218fs and p.S225N mutants show that these may be loss of function alleles. In the case of p.L235F, the reduction of luciferase activity occurred in only two of the four concentrations tested, suggesting that it may not be disease-causing.

To investigate the effect of heterozygous loss of functional alleles, we performed luciferase assays with cotransfections of WT and mutant NRL constructs. Although we tested WT with several NRL mutants (p.S50T, p.P31S, p.E63K, p.A76V, p.G122E, and p.L160P) at various DNA amounts using the rhodopsin promoter, we did not observe significant changes in transcriptional activity except with p.L160P (data not shown). Hence, we do not believe that NRL heterozygote loss of function alleles can cause disease, and that loss of function of both NRL alleles (e.g. p.L75fs/p.L160P) is required, consistent with previous clinical findings [Nishiguchi et al., 2004]. Similarly, homozygous loss-of-function mutations in NR2E3 cause enhanced S-cone syndrome/ARRP [Gerber et al., 2000; Haider et al., 2000]. It is possible that heterozygotes with mutations in NRL are present in RP patients with digenic diallelic or digenic triallelic inheritance pattern. This kind of inheritance has been observed in other forms of RP [Kajiwara et al., 1994; Katsanis et al., 2001]. However, this would likely be a rare event and further work is required to test this hypothesis.

The morphogenesis and function of photoreceptors appear to be under strict genetic control since mutations in a large number of genes can lead to retinal and macular dystrophies. In animal models, both over- and underexpression of rhodopsin have been shown to cause photoreceptor cell death [Humphries et al., 1997; Tan et al., 2001]. Given that NRL plays a key role in rod photoreceptor development and maintenance, its activity must be controlled stringently. Control of NRL activity may provide a useful means to modulate its function by signaling molecules. We propose that phosphorylation and possibly other posttranslational modifications mediate NRL’s interaction with other transcription regulatory proteins, and this in turn affects the nature of transcriptional complexes and target genes during photoreceptor development and in mature rods.
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