Cellular Expression and siRNA-Mediated Interference of Rhodopsin cis-Acting Splicing Mutants Associated with Autosomal Dominant Retinitis Pigmentosa

Imma Hernan,1 María José Gamundi,1 Ester Planas,1,2 Emma Borràs,1 Miquel Maseras,3 and Miguel Carballo1

PURPOSE. To investigate the cellular expression of cis-acting splicing mutations in the rhodopsin gene (RHO) that lead to autosomal dominant or recessive retinitis pigmentosa (adRP/arRP) and the role of nonsense-mediated mRNA decay (NMD) in its pathogenic mechanism. To design a potential therapeutic RNAi-based suppression strategy for cis-acting adRP splicing mutants.

METHODS. Cells were transfected with genomic constructs encoding the human wild-type (WT) and c.531-2A>G, c.936+1G>T, c.937-1G>T and c.745G>T RHO mutants. Total RNA was quantified by RT-PCR and protein was analyzed by immunocytochemistry. Three small interfering (si)RNAs directed against adRP mutant transcripts were designed and assayed in COS7 cells.

RESULTS. The RHO cis-acting splicing mutations causing adRP, c.531-2A>G and c.937-1G>T, induce cryptic splicing. In contrast, the c.936+1G>T mutation, which causes arRP, results in exon skipping. Although the c.531-2A>G and c.745G>T RHO sequence predicted a premature termination codon (PTC) that should be a target for NMD, these mutant proteins were detected in transfected cells. The siRNAs designed to interfere with adRP mutants silenced the corresponding mRNA with varying efficiency.

CONCLUSIONS. Although two RHO mutations that cause different RP phenotypes were the target for the NMD mechanism, a fraction of mutant RNA transcript may circumvent the NMD mechanism and be translated into protein. Thus, different levels of mutant protein may be necessary to trigger the RP phenotype. The findings demonstrate the potential use of siRNA to interfere with cis-acting splicing RHO transcripts. However, limitations in the mutation sequence and incomplete transcript elimination should be considered in a therapeutic approach for adRP. (Invest Ophthalmol Vis Sci. 2011; 52:3723–3729) DOI:10.1167/iovs.10-6933

The splicing process, an essential step in eukaryotic gene transcription, removes introns from pre-mRNA precursors to generate mature mRNAs. The removal of introns from pre-mRNAs requires multiple cis-acting elements that are located in both the intronic and exonic sequences of the genes.1,2 Mutations in cis-acting elements have been reported in almost all genes associated with monogenic diseases, and it has been estimated that nearly 15% of known point mutations causing human genetic diseases affect the splicing process.3-5

Retinitis pigmentosa (RP) constitutes a heterogeneous group of inherited degenerative retinal diseases6,7 characterized by progressive loss of photoreceptor function, resulting in night blindness, reduced peripheral vision, decreased visual acuity, abnormal retinal electrophysiology, and pigmentary retinopathy. RP displays all three modes of Mendelian inheritance: autosomal dominant (adRP), autosomal recessive (arRP), and X-linked (XL RP). Mutations in at least 14 different genes have been associated with adRP,8,9 but mutations in the rhodopsin gene (RHO; OMIM 180380, accession ID U19742) are the most prevalent cause of adRP worldwide (Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/Omim/ National Center for Biotechnology Information [NCBI], Bethesda, MD).

More than 30 different RHO mutations, accounting for 15% of adRP in the Spanish population, have been identified.10 Two of these mutations, c.531-2A>G and c.937-1G>T, are single-nucleotide substitutions that affect the splicing signals of the intron 2/exon 3 and intron 4/exon 5 boundaries, respectively.11,12 These splicing mutations are associated with complete penetrance of adRP. A similar type of mutation in the flanking sequences of exon 4 (c.936+1G>T) has been found to cause arRP.13,14 Recently, using minigenes, we reported that the adRP-causing RHO c.531-2A>G and c.937-1G>T mutations abolish the canonical splicing signals and produce aberrant mRNA splicing products, whereas the RHO c.936+1G>T cis-acting splicing mutation that causes arRP results in the skipping of exon 4.15 Aberrant splicing is a common source of premature termination codons (PTCs) that generate truncated proteins; nonsense-mediated mRNA decay (NMD) targets mRNAs harboring PTCs for degradation in eukaryotic cells.16-18 A recent study14 showed that adRP-causing mRNAs harboring nonsense mutations of PRPF31 are targeted by NMD. Thus, for these PRPF31 mutations, a mechanism of haploinsufficiency, rather than a dominant negative effect, is proposed as a cause of pathogenic RP.19-22

The general consensus is that lack of function of mutant RHO products is associated with recessive forms, whereas dominant cases are most likely caused by gain-of-function (dominant negative effect) of mutant RHO protein. Thus, some reported stop codon and splice site RHO mutations have a low or mild RP phenotype incidence among heterozygous carriers,23 and some of these mutants have been considered similar to recessive null alleles.24 It can be hypothesized that these null
alleles generate a transcript with PTCs that promote a NMD mechanism that may reduce or suppress its transcriptional RNA product, reducing or avoiding a putative dominant negative effect of these mutants in the retina.

It is unknown whether the cis-acting splicing RHO mutations c.531-2A>G and c.937-1G>T lead to the RP phenotype through targeting by NMD, or whether they are translated as dominant negative mutant RHO proteins. Most of the RHO mutations reported in adRP patients suggest a dominant negative effect.\textsuperscript{25,26} For this class of mutation, small interfering (si)RNA-mediated mRNA interference\textsuperscript{27} is a possible therapeutic approach. In this scenario, the mutant RHO mRNA is eliminated by a sequence-specific siRNA that preserves the mRNA of the wild-type allele.\textsuperscript{26,29} As cis-acting splicing mutations may introduce intronic sequences and/or new junctions between two exons in mRNA, these mutant sequences are potential targets for a specific siRNA.

In the present study, we investigated the expression and NMD mechanism of mutant RHO products generated by cis-acting splicing mutations that cause RP and a stop codon mutant that causes arRP. We discuss the utility of the RNA interference of these cis-acting splicing RHO mutants as a therapeutic approach.

**Material and Methods**

**Plasmid Construction**

Genomic clones of wild-type rhodopsin (pCI-RHO WT) and mutants c.531-2A>G (pCI-RHOg.A3811G), c.745G>T (pCI-RHOG.G41435T), and c.936+1G>T (pCI-RHOg.G51677T) were obtained from Exon Biosystems (San Diego, CA) by cloning full-length human RHO (from nucleotide 200 to 5330 of genomic RHO; Genbank U49742; http://www.ncbi.nlm.nih.gov/Genbank; NCBI) into the pCI-neo eukaryotic expression vector (Promega, Madison, WI). Mutant constructs were created by site-directed mutagenesis of the pCI-RHO WT construct. The coding region sequence of each construct was verified by StabVida (Oeiras, Portugal) by dye termination chemistry (Big Dye terminator (Oeiras, Portugal) on a DNA sequencer (3730XL; Applied Biosystems, Inc.

**Cell Culture and Transfection Conditions**

The COS7 and HeLa cell lines were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and cultured in DMEM (COS7) or MEM (HeLa) supplemented with 10% FBS Gold, 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA LabClinics; Barcelona, Spain). Cells were grown to 90% confluence and then subcultured in 1:3 to 1:10 dilutions of trypsin-EDTA.

Cells grown to 90% confluence were transfected with the pCI-neo vector carrying mutant or WT RHO plus siRNA as required, using a lipofection transfection reagent (Lipofectamine 2000; Invitrogen, Barcelona, Spain).

**RT-PCR Analysis of RHO Splicing Products**

Cos7 cells cultured in six-well plates were transfected with genomic constructs encoding the human WT and mutant RHO (Lipofectamine 2000; Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, total RNA was isolated (RNeasy Plus Mini Kit; Qiagen, Izasa, Barcelona, Spain). Two microliters of each RNA sample were used in reverse transcription (RT) by incubation with 50 picomoles anchored-oligo(dT)\textsubscript{18} primer and 10 U reverse transcriptase (Transcriptor; Roche, Barcelona, Spain). PCR of RHO transcripts was performed using Taq polymerase (EcoTaq; Ecogen, Barcelona, Spain) and 5'-AAGCTGACACTCGAGCCACGGGTCAAGAAAG-3' as the forward primer and 5'-GAGCATTATGTCGACAGTTGTTAAGGATGGGAGAC-3' as the reverse primer. Conditions for PCR were 5 minutes at 94°C; followed by 50 cycles of 1 minute at 94°C, 1 minute at 58.5°C, and 1 minute at 72°C, and a final 10 minutes at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis. Direct genomic sequencing was performed to analyze any differences between WT and mutant RHO splicing products.

**Immunocytochemistry**

Approximately 24 hours after transfection of full-length RHO constructs encoding WT and mutant proteins (Table 1), COS7 cells were fixed in 4% paraformaldehyde for 20 minutes, then permeabilized in 0.1% Triton X-100 for 15 minutes at room temperature. The cells were blocked for 1 hour at room temperature with 10% fetal serum before incubation with antibodies. They were first incubated in primary antibody for 2 hours, followed by a 1-hour incubation with conjugated secondary antibodies. In the last wash, the nuclei were stained with DAPI and the cells were mounted on slides with coverslips for microscopic analysis. The cells were examined with a fluorescence microscope (Eclipse 50i, Nikon; Izasa, Barcelona, Spain).

The antibodies used were the mouse monoclonal 1D4 RHO antibody at 1:1000 dilution (Abcam; Cambridge, UK), and the mouse monoclonal RET-P1 RHO antibody at 1:100 dilution (Abcam). For the secondary antibody, we used a goat polyclonal antibody to mouse IgG, conjugated with alkaline phosphatase, at a 1:500 dilution (Abcam).

**Western Blot Analysis**

Full-length RHO constructs were transiently expressed in culture cells. Two days after transfection, the total protein was extracted (Qproteome Mammalian Protein Prep kit; Qiagen).

Proteins were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes and blocked overnight in 1× TBST (20 mM Tris, 500 mM NaCl [pH 7.5], 0.1% Tween 20) containing 5% nonfat dry milk. Membranes were probed with the primary mouse monoclonal 1D4 RHO antibody (1:1000; Abcam). Next, the blots were washed three times for 10 minutes in 1× TBST at room temperature, and subsequently incubated with an alkaline phosphatase-conjugated anti-mouse IgG (1:500; Abcam) in 1× TBST containing 5% milk, followed by washing, as described. The protein bands were visualized with NBT/BCIP (Ready-to-Use NBT/Biofilm Plates; Roche).

**NMD Inhibition**

To investigate whether rhodopsin transcripts are subject to regulation by NMD, we indirectly inhibited NMD by treating HeLa cells with cycloheximide (CHX; Sigma-Aldrich, Madrid, Spain), a general protein translation inhibitor.\textsuperscript{30} Cells were cultured in six-well plates at a density of 1.5 × 10\textsuperscript{5} cells/mL in serum-free medium. Twenty-four hours after transfection of RHO constructs, the cells were incubated at 37°C

### Table 1. Rhodopsin Proteins Predicted from RHO Mutants Containing Aberrant Splice Sites

<table>
<thead>
<tr>
<th>Mutation at CDNA Level (GenBank U49742)</th>
<th>Normal Splice Site</th>
<th>Aberrant Splice Site</th>
<th>Protein Change Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.531-2A&gt;G</td>
<td>g.3811-3812</td>
<td>a) g.3711-3712 (intronic)</td>
<td>Arg177_Tyr178insAspGluMet, fsX181</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) g.3956-3957 (exonic)</td>
<td>Arg177Ser fsX214</td>
</tr>
<tr>
<td>c.936+1G&gt;T</td>
<td>g.4335-4336</td>
<td>Skipping of exon 4</td>
<td>Del Ala243, Gln312</td>
</tr>
<tr>
<td>c.937-1G&gt;T</td>
<td>g.5166-5167</td>
<td>g.5205-5206 (exonic)</td>
<td>Del Phe313_Lys325</td>
</tr>
</tbody>
</table>
in a humidified atmosphere containing 5% CO₂ in air, in the presence or absence of 200 μg/mL CHX for 5 hours before RNA extraction. RHO transscripts were quantified by RT-PCR.

Design and Screening of siRNAs in Cultured Cells
We designed siRNAs targeting the transcriptional products obtained from pCI-RHOG.A3811G and pCI-RHOG.G5167T (Table 2), using the Sfold algorithm. Duplexes of the designed siRNAs were purchased from Ambion, Inc. (Madrid, Spain).

To test siRNAs in vitro, we co-transfected 2 × 10⁵ COS7 cells that had been cultured overnight in a six-well plate with synthetic siRNA (50 picomoles) and recombinant pCI-neo vector expressing WT or mutant RHO (1 μg). An irrelevant siRNA was used as a control. Each well was transfected with 2.5 μL reagent (Lipofectamine 2000; Invitrogen). Twenty-four hours after transfection, total RNA was isolated (RNeasy Plus Mini Kit; Qiagen). Specific quantification of different RHO transcripts was performed on a thermal cycler (LightCycler FastStart DNA Master SYBR Green I Kit; Roche). Amplification of β-microglobulin was used as an endogenous internal control to normalize gene expression. Primers and conditions used for qRT-PCR analysis are available on request.

RESULTS
Splicing Assays of WT and cis-Acting Splicing Mutant Genomic RHO Constructs
We previously characterized the RNA products resulting from RHO minigene constructs carrying cis-acting splicing mutations. In the present study, we cloned the human WT full-length RHO gene (nearly 6 kb) in an expression vector and used site-directed mutagenesis to generate the arRP-associated c.936+1G>T mutation, as well as the two previously identified cis-acting splicing mutations (c.531-2A>G and c.937-1G>T) associated with adRP (Fig. 1A). We transiently transfected these constructs into COS7 cells, isolated the total RNA, and subjected the total RNA to RT-PCR analysis with primers amplifying exons 1 to 5 (to obtain a DNA fragment with the full RHO coding sequence; Fig. 1B). PCR products, which reflected variations in splicing, were resolved and analyzed by gel electrophoresis (Fig. 1C). DNA bands corresponding to the different RT-PCR products were isolated and sequenced, revealing correct splicing of the WT RHO genomic construct, whereas each mutation in the three mutant genomic constructs abolished the splicing signal. Thus, in the c.531-2A>G and c.937-1G>T mutant constructs, the canonical splice sites were abolished, and the aberrant intronic and exonic splice sites were used. In contrast, the c.936+1G>T mutation resulted in a total skip of exon 4 (Fig. 1B). We obtained identical results when RHO constructs were transfected in HeLa cells (data not shown). The results obtained with the full-length RHO genomic constructs are equivalent to those obtained previously with RHO minigenes, except that electrophoresis of RT-PCR products from the RHO genomic constructs do not show the intermediate splicing products (due to lack of complete intron removal) observed in RHO minigene constructs.

Analysis of NMD of Transfected RHO Mutants
The RHO c.531-2A>G cis-acting splicing mutation abolishes a canonical splice site, generating two alternative splicing products (Fig 1B), with each stop codon located more than 50 nucleotides be-
yond the proximal 5′ splicing signal. Thus, the transcriptional products of RHO mRNA harboring the c.531-2A>G mutation may be subject to NMD (reviewed in Ref. 19).

As a control test of the NMD mechanism in our cultured HeLa cells, we analyzed the efficiency of NMD of the -globin gene. We transiently transfected cells with two constructs (kindly provided by Lynne E. Maquat, University of Rochester, NY) carrying either the WT -globin gene or the NS39 nonsense mutation (PTC at codon 39 within exon 2). To normalize the results with respect to the transfection efficiency, we cotransfected the constructs with an empty pCIneo vector.

To study the link between RHO and the NMD mechanism, we transfected HeLa cells with one RHO mutant construct containing the c.531-2A>G mutation, or the previously reported c.745G>T (p.Glu249X) mutation that generates a PTC in RHO exon 4 and that we used as the NMD control. This nonsense mutation has been associated with arRP. We measured the levels of the -globin and RHO transcripts in transfected HeLa cells, with or without CHX. The level of mRNA transcribed, carrying either the normal sequence or the truncated mutants, was quantified by RT-PCR and normalized to the mRNA level of neomycin transcripts. After CHX treatment, a significant increase in the level of NS39 transcript was observed in HeLa cells (Fig. 2A). A similar effect on the level of c.531-2A>G and c.745G>T RHO transcripts in this cell line was observed (Fig. 2B), demonstrating that both mutants are targets of NMD.

**Protein Expression of RHO Mutants**

To investigate whether the cis-acting splicing RHO mutants are translated into protein products, COS7 cell lines were transfected with full-length RHO gene constructs, and immunocytochemistry analysis using anti-RHO antibodies was performed. Notably, the results confirm the translation of the RHO c.531-2A>G mutant (Fig. 3). While the RET-P1 antibody, which recognizes the N-terminal region of RHO, recognizes the mutant protein, the antibody against the C terminus of RHO (1D4) failed to detect c.531-2A>G mutant products. This result is consistent with our prediction that these translated proteins lack the C-terminal RHO region (Table 1).

The predicted protein from the c.937+1G>T mutant lacks the sixth and seventh transmembrane domains, whereas we predict that translation of the c.937-1G>T mutant results in a RHO protein with a 13-residue deletion near the C terminus (Table 1). Both predicted proteins conserve both the N- and
C-terminal regions. As expected, both mutants were translated in COS7 cells and detected by both antibodies (Fig. 3). RHO protein carrying the c.745G>T nonsense mutation was also detected with the RET-P1 antibody. However, this protein species could not be detected with 1D4 antibody, as the mutation produces a protein without the C-terminal RHO region. When we analyzed the protein products obtained in HeLa cell transfection, the same results were obtained (data not shown).

The analysis of total protein extracts from transfected COS7 cells by Western blot resulted in highly aggregate protein products of RHO protein. In an attempt to avoid these aggregates, protein extracts were subjected to PNGase F treatment.25 However, no appreciable improvement resulted. The analysis of total protein extracts from transfected COS7 cells and detected by both antibodies was in combination with a secondary alkaline phosphatase-conjugated antibody. A nontransfected COS7 cell lysate and purified bovine RHO were used as negative (−) and positive (+) controls, respectively. MWM, molecular weight marker.

**siRNAs Target RHO cis-Acting Splicing Mutant Products in COS7 Cells**

To selectively interfere with our cis-acting splicing mutant mRNAs, we used the Sfold algorithm to design efficient siRNAs against the different sequences generated by alternative splicing. Two different siRNAs, R3811B1 and R3811B5, specifically designed to target one of each of the mRNA-splicing products generated by the c.531-2A>G mutant (Fig. 5A). The R3811B1 siRNA efficiently targeted the corresponding mRNA that contained intronic sequences of RHO. The R5167 siRNA was designed to specifically interfere with mRNA product b, generated from the c.531-2A>G mutant. Although the RT-PCR results indicated high interference of the specific mRNA target, levels of WT mRNA also decreased.

The R5167 siRNA, designed to specifically interfere with the mRNA product of the RHO c.937-1G>T mutant, was assessed for its ability to reduce mutant and WT RHO mRNA levels. The RT-PCR results (Fig. 5B) demonstrated that R5167 efficiently silenced the mutant mRNA but had no impact on WT levels.

**DISCUSSION**

The predicted translation of mRNA containing the c.531-2A>G RHO mutation results in two proteins, each with a PTC (Table 1). The PTCs in the translated proteins predict that an mRNA decay mechanism is triggered, preventing the mRNAs from being translated into proteins. However, immunocytochemistry results from transiently transfected COS7 and HeLa cells showed protein expression of the WT as well as the two cis-acting splicing mutants of RHO. In our experiments, the mRNA encoding the c.531-2A>G mutant was as low as completely, by NMD when the mutant construct was transfected in HeLa cells and the truncated protein were expressed.

In the retina, if the c.531-2A>G mutant products are expressed, the RP phenotype would be caused by a dominant negative or deleterious gain-of-function effect, rather than by haploinsufficiency due to a null allele. Thus, the RHO mutation c.745G>T, also subject to NMD, does not produce a dominant negative effect. This mutation, which encodes a presumable nonfunctional protein, lacking the C terminus, is detectable in
heterozygous RP-unaffected carriers and causes the RP phenotype in homozygous patients. The presumed reduction in RHO c.745G>T mutant by NMD seems to be sufficient to avoid the RP phenotype in heterozygous carriers, although slightly reduced rod ERG responses in these individuals have been reported. However, our experiments in transiently transfected HeLa cells with the RHO constructs c.531-2A>G and c.745G>T did not allow detection of significant quantitative differences in NMD function.

Translation of the RHO c.937-1G>T mutant results, by generation of an aberrant splicing site, in a protein that lacks 13 amino acid residues. In contrast, transcription of the RHO c.936+1G>T does not use aberrant splice sites and results in the skipping of exon 4, causing an arRP trait.

Different levels of mutant protein in the retina could be necessary to trigger the RP phenotype. However, in each case, the reduced levels of protein could be explained by lower production of the mutant product due to partial NMD function, a differential splicing rate between normal and aberrant splicing signals, or compromised stability of the truncated proteins.

We conclude that RHO cis-acting splicing mutations abolished the canonical splice site, with some showing aberrant splicing sites. Some of these mutations may generate in-frame deletions—for example, by exon skipping or PTCs that may elicit NMD—resulting in a decrease in the mutant transcript in the photoreceptor cell, leading to a mild dominant or recessive RP phenotype in heterozygous carriers or the mimic the RP phenotype caused by a null recessive allele in recessive RP carriers.

The pathogenic mechanisms of most of the RHO mutations in heterozygous adRP patients occur via gain of function of the mutant allele. A rational therapeutic approach for these cis-acting splicing mutations would include the suppression of such a mutant allele. Recently, RNAi technology has demonstrated potent suppression of gene expression. Thus, interference of mRNA cis-acting splicing mutants by a specific siRNA that does not affect the expression of WT RHO may mimic the RP phenotype caused by a null recessive allele in heterozygous carriers or the Rho<sup>-/-</sup> mouse model.

In the present study, we used siRNAs to specifically suppress the mutant c.957-1G>T mRNA product, as well as one of the c.531-2A>G mutant products (Fig. 5). However, the other c.531-2A>G product could not be specifically suppressed, because the siRNA designed not only interferes efficiently with the mutant RHO mRNA but also abolishes WT RHO expression. This fact may compromise the design of an efficient siRNA to interfere specifically in some RHO mutations. To circumvent this limitation, a novel strategy consisting of RNAi suppression and gene replacement has been proposed. This strategy involves the suppression of both alleles (WT and mutant) of a target gene by RNAi and simultaneous replacement with a modified functional sequence (degenerate) gene that evades suppression. This approach has recently been reported for the RHO gene.

Our results suggest that extensive suppression of mutant alleles of RHO may be necessary in a therapeutic approach, as minor mutant mRNAs could cause a pathogenic phenotype. Thus, in the case of RHO, the suppression and replacement approach seems to be the more productive method.

**Acknowledgments**

The authors thank Ian Johnstone for the English revision and editing of the manuscript.

**References**


24. Rosenfeld PR, Cowley GS, McGee TL, Sandberg MA, Berson EL, Dryja TP. A null mutation in the rhodopsin gene causes rod
photoreceptor dysfunction and autosomal recessive retinitis pig-

25. Rajan RS, Kopito RR. Suppression of wild-type rhodopsin matura-
tion by mutants linked to autosomal dominant retinitis pigmento-

26. Mendes HF, Cheetham ME. Pharmacological manipulation of gain-
of-function and dominant-negative mechanisms in rhodopsin reti-


29. Ohnishi Y, Tamura Y, Yoshida M, Tokunaga K, Hohjoh H. En-


31. Zhang J, Sun X, Qian Y, Maquat LE. Intron function in the non-


