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In vitro Analysis of Ribozyme-mediated Knockdown of an ADRP Associated Rhodopsin Mutation

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1 Introduction

Retinitis Pigmentosa (RP) is a group of retinal degenerative diseases that are characterized mainly by the loss of rod photoreceptor cells. RP can be sub divided into 3 classes, autosomal dominant, autosomal recessive, and X-linked RP, where the mutant gene exists on the sex chromosome (X) (Hartong et al., 2006; Wang et al., 2005). Mutations in rhodopsin are the most common cause of the autosomal-dominant form of RP (ADRP). More than 100 mutations in rhodopsin account for approximately 30% of ADRP cases with varying severity of visual impairment (Dryja et al., 1991). Based on *in vitro* studies (Sung et al., 1993; Sung et al., 1991), rhodopsin mutations that are similar to wild-type in terms of expression levels, folding and formation of functional photopigment are considered Class 1 mutations. On the other hand Class 2 mutants are those in which the opsin protein is inefficiently transported or is retained in the endoplasmic reticulum (ER). The mutation of Proline to Histidine at the 23rd amino acid of rhodopsin (P23H) is a Class 2 mutation. The *Xenopus laevis* in vivo model for P23H (Tam and Moritz, 2006) shows that the protein is misfolded and that is accumulated in the inner segment (IS) causing ER overload and cellular stress thus supporting its categorization as Class 2. It has been shown that the P23H substitution reduces the binding capacity for 11-*cis* retinal and causes a defect in glycosylation (Noorwez et al., 2004). The first symptom of ADRP associated with the P23H mutation is impaired night vision that appears between 10 to 30 years of age. The majority of these individuals became blind by the age of 60.

Gene therapy is one of the most promising treatments for inherited retinal diseases. At the beginning it was a challenge to achieve successful gene therapy in the retina due to the lack of cell specific and efficient targeting as well as the lack of a delivery method that would cause minimum damage to the eye. Additionally, there were no specific animal models that closely mimicked the human disease presentations. So far, two types of approaches have been considered for gene therapy in ADRP animal models. The first is delivery of genes for neurotrophic factors such as GDNF (McGee Sanftner et al., 2001) and antiapoptotic proteins such as XIAP (Petrin et al., 2003) to prevent the ongoing death of the photoreceptors. The second approach is to block the synthesis of mutant proteins and increasing the ratio of wild-type vs mutant rhodopsin to delay the onset of retinal degeneration (Drenser et al., 1998; LaVail et al., 2000). For instance, Lewin and his colleagues used AAV2 to deliver ribozymes that cleaved the P23H rhodopsin mRNA and showed an effective treatment in a rat model of ADRP (LaVail et al., 2000).

Recently various reports demonstrate the robust efficacy of RNA interference (RNAi) in animal models of human disease and support the RNAi based new therapeutic approach as one with the potential to change the treatment of human disease (Bumcrot et al., 2006). RNAi has been

used to silence P23H human rhodopsin alleles by 94.9% in human embryonic retinoblasts (Cashman et al., 2005) thus providing further evidence that this approach is a viable one to treat specific human disease causing mutations.

2 Animal Model

Genetic diseases can result from both the expression of a mutant protein or the failure to express its normal form. The development of molecular biology and transgenic mouse technology has enabled researchers to generate multiple relevant animal models for the study of genetic eye diseases (including P23H) (Nour and Naash, 2003). The functional and structural defects in these models can closely mimic the human disease phenotypes. We have generated three rhodopsin transgenic disease models: one carrying the P23H mutation (Late onset ADRP) (Naash et al., 1993; Naash et al., 1996a, b; Wang et al., 1997; Wu et al., 1998) one carrying the Δ I-255/256 deletion which causes early onset ADRP (Penn et al., 2000), and one with the G90D mutation which causes congenital stationary night blindness (CSNB) (Naash et al., 2004). These animal models allow us to conduct the extensive biochemical, histological and physiological studies which necessarily underlie the development of effective therapeutic interventions.

3 Gene Therapy

Since the P23H retinal degeneration is inherited as an autosomal dominant fashion and very often caused by the product of a single mutant allele that may interfere with the function of the protein encoded by the wild-type allele, a reduction in the expression of the mutant allele may prevent or delay the disease phenotype. While currently no therapies exist for the treatment of ADRP, gene therapy may serve the best and direct way for future therapeutic intervention. Researchers have investigated ribozyme technologies to reduce the expression of mutant gene products. It has been shown that ribozyme therapy can successfully reduce the P23H mutant protein in the rat retina (Fritz et al., 2004; Gorbatyuk et al. M., 2007; LaVail et al., 2000; Lewin et al., 1998).

3.1 Ribozymes

The discovery of naturally occurring RNA molecules which were able to regulate gene expression led to the idea that oligonucleotides could be used for gene therapy (Blake et al., 1985; Zamecnik and Stephenson, 1978). These discoveries consequently generated the hypothesis that oligonucleotides could be designed to down-regulate the expression of genes responsible for pathogenesis. At about the same time, the study of RNA processing led to the finding of naturally occurring RNA sequences that possessed catalytic properties (Inoue et al., 1985). These RNA molecules were called ribozymes, an abbreviation for ribonucleotide enzyme, stressing the ability of these RNA molecules to act as endonucleases. Ribozymes form base pair specific complexes with their RNA substrates and are able to catalyze the hydrolysis or phosphoryl exchange at a phosphodiester bond. Furthermore, the observation that ribozymes could be designed to act in *trans* led to the development of antisense technology. In addition, ribozymes do not rely on the host cellular machinery to degrade their substrate, and they can be classified into different groups based on their catalytic activities. The three main groups of ribozymes are the self-splicing introns, RNase P, and the small self-cleaving ribozymes. Each type of ribozyme is being examined for its possible use as a genetic therapy, but the hammerhead and hairpin ribozymes have received most attention for potential clinical applications. The hammerhead ribozyme recognizes the substrate sequence on either side of an NUX cleavage site by means of two flanking arms that hybridize to form helices III and I (Fig. 1). Cleavage occurs at 3' of the NUX consensus sequence, where N = any nucleotide, and X = any nucleotide except G. Cleavage sites do not all show the same efficiency. In general, GUC is the most efficient cleavage site, followed by CUC, UUC, and AUC. The rest of the

cleavage sites are cleaved at least ten-fold more slowly than the GUC site (Shimayama et al., 1995).

The hairpin ribozyme binds its substrate to form a structure with four helices and two loops. The arms of the hairpin ribozyme hybridize to the substrate molecule to form a six base pair helix 1 and a four base pair helix 2 (Fig. 1). The sequence of these arms can be manipulated to allow binding to a variety of RNA substrates. Loop A contains the BNGUC target sequence required for cleavage where B = G, C, or U and N is any nucleotide (Hampel et al., 1990). There are no conserved nucleotides in any of the helices, although a G in the substrate portion of helix 2 adjacent to loop A leads to an increased rate of cleavage (Joseph and Burke, 1993). There are a number of conserved nucleotides found in loop A and loop B, which fold on top of one another to form the catalytic core (Esteban et al., 1998).

3.2 VPP Transgenic Mouse Models of ADRP

The P23H mutation in the opsin gene is one of the most prevalent mutations found in the United States, and accounts for over 12% ADRP patients (Wang et al., 2005). To examine the link between the P23H genotype and its phenotype, we have created a transgenic line named VPP that expresses mutant rhodopsin containing three substituted amino acids in the N-terminus [Val20→Gly (V20G), Pro23→His (P23H) and Pro27→Leu (P27L)] (Naash et al., 1993). These mice express equal amounts of mutant and wild-type transcripts, and develop progressive photoreceptor degeneration (Goto et al., 1996; Wang et al., 1997).

Retinal degeneration in the VPP mice appears as early as postnatal day 20 (Fig. 2) and continues to degenerate in a light-dependent manner (Wang et al., 1997). Several studies have shown that the P23H protein can traffic to the outer segments of rod photoreceptors only in the presence of the wild-type rhodopsin and can bind to the chromophore, 11-*cis* retinal (Qtaishat et al., 1999; Wu et al., 1998). Since it cannot transport to the outer segment on its own, it failed to support outer segment formation in the rhodopsin null background ($Rho^{-/-}$) (Fig. 2). The VPP protein in the $Rho^{-/-}$ background was found trapped in the ER and the animals showed no scotopic ERG a- wave and accelerated photoreceptor degeneration. Interestingly, this degeneration occurs more rapidly than degeneration associated with rhodopsin knockout alone (Frederick et al., 2001).

A number of factors make the VPP mouse model ideal for the evaluation of the efficiency of ribozymes *in vivo*. First, the retinal degeneration in these animals is slow, which gives adequate time for the ribozyme to have an effect. Second, the level of mutant message is equivalent to that of the wild-type, a situation similar to that seen in patients. Consequently, injecting the right eye of a mouse with the ribozyme construct and the left eye with a construct that acts as a control should allow an accurate determination of the activity of a ribozyme *in vivo*. The change in ERG can be used to measure the efficiency of a ribozyme without having to sacrifice mice that have been injected with the treatment. Thus, the effects of a ribozyme can be followed over time. This in turn will aid in the design of future ribozymes with increased therapeutic activity. This will be especially important for ribozymes designed to treat autosomal dominant genetic diseases.

3.3 Design of Ribozymes for the Treatment of P23H Associated ADRP

In ADRP, expression of the mutant protein results in retinal dystrophy (Dryja et al., 1990). The hypothesis underlying ribozyme therapy for this model is that decreasing the expression of the mutant protein will delay or prevent the disease. The success of ribozyme therapy depends on the identification of an RNA target site within the mutant allele. The hammerhead ribozyme is able to cleave an NUX target sequence, but the efficiency of the cleavage reaction can vary by as much as 100-fold depending on the precise cleavage triplet. The most efficient cleavage

triplet is a GUC sequence followed by CUC, which is five-fold less efficient, and UUC, which is 10 times less efficient. The hairpin ribozyme has slightly different target site requirements than the hammerhead ribozyme. The target cleavage site of the hairpin is less flexible. Mutational analysis demonstrated that only the GUC triplet is cleaved efficiently (Hampel et al., 1990).

Two strategies were used to ensure that a ribozyme was able to specifically cleave the mRNA of the mutant but not the wild-type. The first approach was to identify a mutation that creates a cleavage triplet target in the mRNA of the mutant gene. The second strategy was to identify a mutation in close proximity to the cleavage triplet in the substrate. These mutations allow cleavage of the mutant mRNA but not the wild-type mRNA because the mismatched base pairs disrupt the formation of the catalytic core of the ribozyme (Grasby et al., 1995). Werner and Uhlenbeck (Werner and Uhlenbeck, 1995) have shown that a mismatch in any of the first four innermost nucleotides of helix III of a hammerhead ribozyme prevented cleavage. Only a mismatch in the innermost nucleotide of helix I prevented cleavage.

One hammerhead and three different hairpin ribozymes, HP1, HP2 and HP3 were designed to target the P23H message (Table 1). The specificity of the cleavage reaction catalyzed by these ribozyme was primarily due to the GUC cleavage triplet found in the transgene sequence of the VPP model but not in the wild-type rhodopsin gene (Naash et al., 1993). The hammerhead and hairpin ribozymes were both designed against the VPP transgene because ribozymes cleave different substrates with different catalytic efficiencies. For instance in the Tang et al study (Tang et al., 1994), hammerhead ribozymes cleaved the same substrates better than the hairpin ribozymes. The design of the three hairpin ribozymes was different. HP1 is the hairpin ribozyme found in the negative strand of the Tobacco Ringspot virus (Hampel et al., 1990) while the HP2 has a helix 4 with three additional base pairs and a stable loop to stabilize its secondary structure and to increase its catalytic activity. The third hairpin ribozyme, HP3, also has the modifications found in HP2 with the addition of a stem loop between helix 2 and helix 3. This stem loop was reported to increase the ability of the hairpin ribozyme to fold into its proper conformation (Esteban et al., 1998).

3.4 Catalytic Efficiency of the Ribozyme in vitro

All the ribozymes targeting the VPP transgene were able to specifically cleave the mutant oligonucleotide in vitro. HH1 cleaved 10% of their substrate in 24 hours while HP1 cleaved 10% of its substrate by 12 hours showing HP1 was twice as efficient as HH1 and 3–4 times less active than HP2 and HP3, respectively (Fig. 3). The HP2 and HP3 ribozyme cleaves 10% substrate in 5 and 3 hours respectively (Figs. 4A and 4B).

The kinetic experiments were performed with 10 mM transcribed ribozyme and increasing concentrations of substrate. For the HP2 ribozyme, the kinetic reactions were analysed 5 hours after starting the reaction. (Fig. 5A). Eadie-Hofstee analysis was used to calculate V_{max} and K_M by performing linear regression analysis of $1/V_0$ versus $V_0/[S]$ (Fig. 5B)

4 Summary of the Effectiveness of Ribozyme Therapy

Rhodopsin associated ADRP cases are mainly gain-of-function phenotypes and not a haploinsufficiency disorder due to the loss of one allele. Therefore, a knockdown of mutant rhodopsin using ribozyme may be one of the best therapeutic approaches. The first test of the efficacy of ribozymes in mouse retinas was done by Lewin's group (LaVail et al., 2000). Later, they showed rhodopsin (Gorbatyuk et al., 2005) and PDE- γ (γ -subunit of rod cGMP phosphodiesterase) (Liu et al., 2005) specific ribozyme knockdown of their target genes. Very recently the use of ribozyme Rz525 in the P23H transgenic rats was able to reduce mutant transcript by 46% which resulted in a partial rescue of the retinal degeneration. The relative

rescue was 50% for both a-wave and b-wave amplitudes in ribozyme treated eyes when compared to control eye. They also observed an increase in the photoreceptor outer nuclear layer (ONL) thickness (Gorbatyuk et al., 2007). This P23H transgenic animal model allowed accurate in vivo studies to evaluate the efficacy and safety of novel therapies. Such features make these models invaluable tools for the development of potential therapeutic interventions. Since there is a high level of genetic heterogeneity in rhodopsin-linked RP, mutation-independent ribozyme therapy approaches will be highly preferable strategy for future therapy.

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Fig. 2.

Retinal structure in VPP transgenics. Histological evaluation (light microscopy) at postnatal day 20 shows retinal degeneration of the *VPP*^{+/-}/*Rho*^{+/+} mice when compared to non-transgenic *Rho*^{+/+} littermate. Expression of the VPP transgene on the *Rho*^{-/-} background resulted in a faster rate of photoreceptor degeneration and an absence of outer segment structures

Fig. 3.
Time course showing the cleavage rate of the VPP target by the ribozymes

Fig. 4. Cleavage rate of the HP2 (A) and HP3 (B) hairpin ribozyme. The time courses of the cleavage reactions for the mutant and wild-type oligonucleotides are shown

Fig. 5. HP2 hairpin ribozyme multiple turnover cleavage reaction. (A) An image of the autoradiogram of the HP2 multiple turnover cleavage reaction. (B) A graph of the Eadie-Hofstee plot used to calculate the kinetic coefficients of the HP2 ribozyme

Table 1

The following deoxynucleotides were used to clone the ribozymes. Sense Strand – Oligonucleotide which has the same sequence as the ribozyme mRNA. Antisense – Oligonucleotide which has a sequence complimentary to ribozyme mRNA

Ribozyme	Sense strand	Antisense strand
HH1 Hammerhead	5'CTC CGG CCG AAG TCT G	5'GAG CAG CGT CGG AGT TTC GCG CTT TCG CGC TCA TCA GAC TTC GGC CGG AG
HP1 Hairpin	5'GCA GAA TTC AGC GGC CGC ACG AAG TAG AAC CGA ACC AGA GAA ACA CAC G	5'GCC ACG CGT ACC AGG TAA TAT ACC ACA ACG TGT GTT TCT CTG G
HP2 Hairpin	5'GCA GAA TTC AGC GGC CGC ACG AAG TAG AAC CGA ACC AGA GAA ACA CAC G	5'GCC ACG CGT TAC CAG GTA ATG TAC CAC GAC TTA CGT CGT GTG TTT CTC TGG
HP3 Hairpin	5'AGC GGC CGC ACG AAG TAG AAC CGA CCC GAC GAC GTA AGT CGT CCC CAC CAG AGA AAC ACA CG	5'GCC ACG CGT TAC CAG GTA ATG TAC CAC GAC TTA CGT CGT GTG TTT CTC TGG