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The Nature of Dominant Mutations of Rhodopsin and Implications for Gene Therapy

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Abstract

Mutations in the rhodopsin gene are the most common cause of retinitis pigmentosa (RP) among human patients. The nature of the rhodopsin mutations has critical implications for the design of strategies for gene therapy. Nearly all rhodopsin mutations are dominant. Although dominance does not arise because of haploinsufficiency, it is unclear whether it is caused by gain-of-function or dominant-negative mutations. Current strategies for gene therapy have been devised to deal with toxic, gain-of-function mutations. However, analysis of results of transgenic and targeted expression of various rhodopsin genes in mice suggests that dominance may arise as a result of dominant-negative mutations. This has important consequences for gene therapy. The effects of dominant-negative mutations can be alleviated, in principle, by supplementation with additional wild-type rhodopsin. If added wild-type rhodopsin could slow retinal degeneration in human patients, as it does in mice, it would represent a valuable new strategy for gene therapy of RP caused by dominant rhodopsin mutations.

Index Entries: Retinitis pigmentosa; rhodopsin mutations; dominant mutants; mouse expression studies; gene therapy.

Introduction

Retinitis pigmentosa (RP) is a heterogeneous group of hereditary disorders that lead to the progressive loss of retinal function. Overall, RP is the most common inherited disorder that causes blindness, affecting 1/5000 people worldwide *(1).* Clinically, RP patients are affected by night blindness and retinal degeneration, which often begins with the loss of peripheral vision in adolescence and progresses over the ensuing decades to tunnel vision and eventual loss of central vision. The progressive nature of the disease means that treatments to

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delay its time-course may be of immense benefit to patients.

Several therapeutic strategies are being tested in model systems. These include delivery of nutrients and pharmacological agents, introduction of growth factors and survival factors, interference with apoptosis, corrective gene therapy, transplantation of retinal or stem cells, and replacement of retinal function with electronic devices *(2,3).* In this article, we focus specifically on the nature of the dominant mutations in the rhodopsin gene and the implications for gene therapeutic approaches to ameliorate their consequences. Rhodopsin, which is composed of the encoded opsin and its attached chromomore, 11-*cis*-retinal, is the G-protein-coupled receptor that is the primary photon detector in rod photoreceptor cells. Defects in the rhodopsin gene have dramatic effects on retinal health, as revealed by studies in human patients as well as expression studies in mice (Table 1). We draw extensively on results from studies in mice to understand the mutational basis for the dominant phenotype, which is critical for exploiting the full range of options for gene therapy.

Dominant Mutations in the Rhodopsin Gene

Mutations in more than 30 genes have been identified as causes of RP, but the genes responsible for nearly one-half of RP cases have not yet been identified *(3–6).* As a class, mutations in the rhodopsin gene are the most common, accounting for about 10% of all cases of RP *(5,7).* In all, more than 100 different rhodopsin mutations have been identified among RP patients. At this locus, dominant RP mutations are 25 times more frequent than recessive ones. Dominant mutations are spread throughout the gene, affecting virtually all parts of the encoded opsin. The reason that such a high fraction of rhodopsin mutations should be dominant is unclear. The answer may involve the special role that rhodopsin plays in rod photoreceptor cells, or the fact that rhodopsin accounts for nearly 50% of the protein content of the outer segments and 80% of protein in the discs *(8).*

A thorough understanding of the nature of these mutations is important because it defines the range of treatment options. For example, gene therapy directed at recessive diseases usually involves introducing a normal gene to compensate for the nonfunctional alleles. By contrast, dominant mutations present a richer challenge for gene therapy *(3,9).* Dominance can arise through loss-of-function mutations, gain-of-function mutations, and dominant-negative mutations. Some insights can be gained from human patients, but transgenic and targeted expression studies in mice (Table 1) have the capacity to discriminate among these types of mutations.

Loss-of-Function Mutations

Loss-of-function alleles are typically recessive, but they can give rise to dominant inheritance if the remaining normal copy does not express sufficient protein to meet a cell's needs. Dominance resulting from inadequate expression—known as haploinsufficiency—is rare, and for most genes, one copy is good enough. Haploinsufficiency of rhodopsin is clearly not the cause of dominant RP in humans. Two observations in humans support this conclusion. None of the dominant rhodopsin alleles that have been studied appears to be a null mutation. And the only characterized null mutation, E249ter, is a recessive allele *(10).*

Targeted knockouts in mice confirm the theory that expression of a single copy of the rhodopsin gene is sufficient for retinal health (Table 1). Knockout of the mouse rhodopsin gene yields a heterozygous phenotype with healthy, functional retinas that display minimal or no retinal degeneration *(11,12).* In the presence of a null mutation, expression of the good copy of the gene could be upregulated to compensate for the loss. This does not seem to be the case, since expression of the wild-type allele in a knockout heterozygote is about half that in a normal mouse (*11;* Table 1). Thus, the dominant alleles that characterize the rhodopsin

locus must result either from gain-of-function or dominant-negative mutations (Fig. 1).

Gain-of-Function Mutations

Gain-of-function mutations often arise by misexpression of the normal protein in the wrong place or at the wrong time, as occurs in the classic homeotic mutants in *Drosophila (13).* Because dominant rhodopsin alleles are expressed on schedule in mouse rod photoreceptor cells, misexpression seems to be an unlikely basis for the dominant phenotype. Gain-of-function alleles can also arise by alteration of the protein to create a novel activity, which could serve as the basis for dominance at the rhodopsin locus. In principle, the acquired novel function could be highly specific, stimulating some inappropriate process in the rod cell, or relatively nonspecific—gumming up the works, so to speak. Each type of explanation has been offered for particular rhodopsin mutations.

K296E, a dominant rhodopsin mutation found in human patients with RP *(14),* eliminates the lysine residue to which 11-*cis*-retinal is attached. In vitro, K296E constitutively activates transducin—the G protein in the phototransduction pathway *(15).* Constitutive activation of transducin would represent a specific gain-of-function mutation analogous to *ras* mutations, which lock Ras into an active form that stimulates downstream signaling regardless of upstream inputs *(16).* Constitutively activated opsin might reasonably lead to retinal degeneration via continuous stimulation, which happens with long-term exposure to constant light *(17).* In mice, K296E causes retinal degeneration, as it does in humans (*18;* Table 1). However, it does not constantly stimulate the phototransduction cascade because it is phosphorylated and bound by arrestin, which prevents its interaction with transducin *(18).* If K296E is inactivated by arrestin in humans, its novel activity is unlikely to serve as the basis for its dominant phenotype.

P23H—the most prevalent allele among RP patients in the United States—is retained in the ER when expressed in cultured cells *(19).* Analysis of P23H in these cells shows that it aggregates and overwhelms the ubiquitin proteasome system *(20,21).* If P23H behaved in this way in rod photoreceptor cells, it would represent a toxic gain-of-function that could lead to cell death. Many neurodegenerative diseases are also characterized by the accumulation of aggregated, ubiquitinylated proteins, suggesting that RP may share a common pathogenic mechanism with other neurological disorders *(20,22,23).* A nonspecific, toxic gain of function is a very attractive explanation because it could explain the pathology of many, if not all, of the dominant alleles of the rhodopsin gene. But is gain-of-function the correct explanation for dominant RP?

Dominant-Negative Mutations

Dominant RP could also be caused by dominant-negative mutations in the rhodopsin gene. Dominant-negative mutations typically eliminate one or more functions associated with the normal protein *(24).* It is the loss of some activities and the retention of others that gives these mutations their dominant-negative properties. Dominant-negative mutations are usually found in genes whose products form multimeric complexes, either with themselves or with other proteins *(24).* For example, the tumor suppressor *p53* is a transcription activator that functions as a tetramer. Many of the mutant forms of *p53* in cancer cells retain the ability to form oligomers with the wild-type protein, but they poison the complex *(25,26).* Assuming that one copy of mutant *p53* can inactivate a tetramer and that the mutant and normal alleles are equally expressed and assorted, cells that are heterozygous for the mutation would have 16-fold less of the functional complex *(26).*

In the disc membranes of rod outer segments, rhodopsin molecules form dimers that align like eggs in a carton (27; Fig. 1). No functional role has yet been ascribed to these oligomers, and some studies have argued that monomers are the active form in phototransduction *(28).* Nevertheless, there is a growing appreciation of the role of dimerization in the structure and function of many G-protein-coupled receptors *(29),* which

Table 1
Transgenic and Targeted Expression of Rhodopsin in Mice

	Modification			Expression Levels[e]					
Type	transgene	targeted	Mouse *RHO*[d]	*RHO*1	*RHO*2	TG	Total	Retinal Degeneration[i]	Ref.
null		del	−/−	0%	0%		0%[h]	Severe	*11*
null		ins	−/−	0%	0%		0%[g]	Severe	*12*
null		ins	+/−	50%	0%		50%	Minimal	*12*
null		del	+/−	57%	0%		57%[h]	Minimal	*11*
wild			+/+	50%	50%		100%	Minimal	
wild	mrho-wt-29		+/+	50%	50%	30%[g]	130%	Minimal	*31*
wild	mrho-wt-48		+/+	50%	50%	240%[g]	340%	Severe	*31*
wild	mrho-wt-1		+/+	50%	50%	520%[g]	620%	Severe	*31*
wild	hrho-wt (NHR-E)		−/−	0%	0%	100%	100%	Minimal	*39*
wild	hrho-wt (NHR-E)		+/+	50%	50%	100%[g]	200%	Minimal	*32*
wild	hrho-wt (NHR-E)/ hrho-wt (NHR-E)		+/+	50%	50%	200%[g]	300%	Moderate	*40*
wild	hrho-wt (NHR-A)		+/+	50%	50%	500%[g]	600%	Severe	*32*
point	hrho-T17M (V)		+/+	50%	50%	nd	nd	Moderate	*41*
point	mrho-GHL[a]		−/−	0%	0%	15%[h]	15%	Severe	*33*
point	mrho-GHL[a]		+/−	50%	0%	15%	65%	Severe	*33*
point	mrho-GHL[a]		+/+	50%	50%	15%	115%	Moderate	*33,42,43*
point	hrho-P23H-L		+/+	50%	50%	16%[g]	116%	Severe	*32*
point	hrho-P23H-E		+/+	50%	50%	100%[g]	200%	Severe	*32*
point	hrho-P23H-D		+/+	50%	50%	300%[g]	400%	Severe	*32*
point	hrho-K296E-A		+/+	50%	50%	25%[g]	125%	Minimal	*18*
point	hrho-K296E-F2		+/+	50%	50%	100%[g]	200%	Severe	*18*
point	hrho-K296E-G2		+/+	50%	50%	300%[g]	400%	Severe	*18*
point	hrho-K296E-E		+/+	50%	50%	1000%[g]	1100%	Severe	*18*
point	mrho-ETV[b] (B)		−/−	0%	0%	70%	70%	Minimal	*44*
point	mrho-ETV[b] (A)		+/+	50%	50%	23%	123%[f]	Moderate	*44*
point	mrho-ETV[b] (A)/ mrho-ETV[b] (A)		+/+	50%	50%	46%	146%	Severe	*44*
point	mrho-ETV[b] (B)		+/+	50%	50%	70%	170%[f]	Severe	*44*
point	mrho-S334ter		+/+	50%	50%	10%[h]	110%	Minimal	*45*

point	mrho-Q344ter-9		+/+	50%	50%	80%[g]	180%	Moderate	*31*
point	mrho-Q344ter-1		+/+	50%	50%	130%[g]	230%	Severe	*31*
point	mrho-Q344ter-20		+/+	50%	50%	150%[g]	250%	Severe	*31*
point	hrho-P347S (C1)		+/+	50%	50%	25%[g]	125%	Moderate	*40*
point	hrho-P347S (A1)		+/+	50%	50%	100%[g]	200%	Severe	*40*
point	hrho-P347S (C2)		+/+	50%	50%	100%[g]	200%	Severe	*40*
point	hrho-P347S (A2)		+/+	50%	50%	500%[g]	600%	Severe	*40*
GFP		hrhoG[c]	+/G	50%[h]	40%[h]		90%	Minimal	[j]
GFP		hrhoG[c]	G/G	40%	40%		80%	Severe	[j]
GFP		hrhoG(H)[c]	+/G(H)	50%[h]	8%[h]		58%	Minimal	[j]
GFP		hrhoG(H)[c]	G(H)/G(H)	8%	8%		16%	Moderate	[j]

[a] The GHL transgene encodes a rhodopsin with three amino acid changes (V20G, P23H, and P27L) relative to the normal mouse sequence *(42)*. The transgene was designed with the expectation that P23H would be the critical change.

[b] The ETV transgene encodes a rhodopsin with three amino acid changes (D332E, A335T, and A337V) relative to the normal mouse sequence *(44)*. These three changes create an epitope that is recognized by a monoclonal antibody against bovine rhodopsin. None of the individual changes has been identified in human RP patients, and this grouping of amino acids functions perfectly well in bovine rhodopsin. In the context of mouse rhodopsin, however, these changes do not appear to be well-tolerated, giving rise to severe retinal degeneration at modest expression levels. It is for this reason that they have been classified here with point mutations rather than with wild-type.

[c] hrhoG and hrhoG(H) encode human rhodopsin fused at its C-terminus to GFP *(36)*. hrhoG was created by standard homologous targeting, whereas hrhoG(H) was created by segmental replacement using loxH and lox511 sites *(46)*. The two targeted genes differ in their 5′ untranslated regions, which presumably accounts for their different levels of expression.

[d] *RHO* stands for the mouse rhodopsin locus, "+" is the wild-type allele, and "–" is a null allele from ref. *11* or *12*.

[e] *RHO1* and *RHO2* refer to the rhodopsin genes (natural or engineered) at the native mouse rhodopsin locus. TG refers to the transgene and total is the sum of expression from all rhodopsin genes. "nd", "not determined." Rhodopsin levels were quantified where percentages are annotated with superscripts, which refer to the method of quantification. Percentages without superscripts were calculated from measurements in other lines.

[f] Expression determined by ELISA.

[g] Expression determined by reverse transcriptase-polymerase chain reaction (RT-PCR).

[h] Expression determined by Western blot.

[i] Retinal degeneration was classified as minimal, moderate, or severe based on electroretinogram (ERG) measurements or light-microscopic examination of retinal slices. In general, "minimal" is used to indicate that little or no change was evident by 2 mo; "moderate" to indicate that more than 50% of the ONL or ERG response remained at 2 mo; and "severe" to indicate that less than 50% of the ONL or ERG response remained at 2 mo. In several cases, the degree of degeneration at 2 mo has been interpolated or extrapolated from data in the papers. For this reason, comparisons within a paper are more reliable than comparisons between papers. Also, the designations used here may not correspond precisely to those used in the papers.

[j] Unpublished observations by F. Chan, T.G. Wensel, and J.H. Wilson.

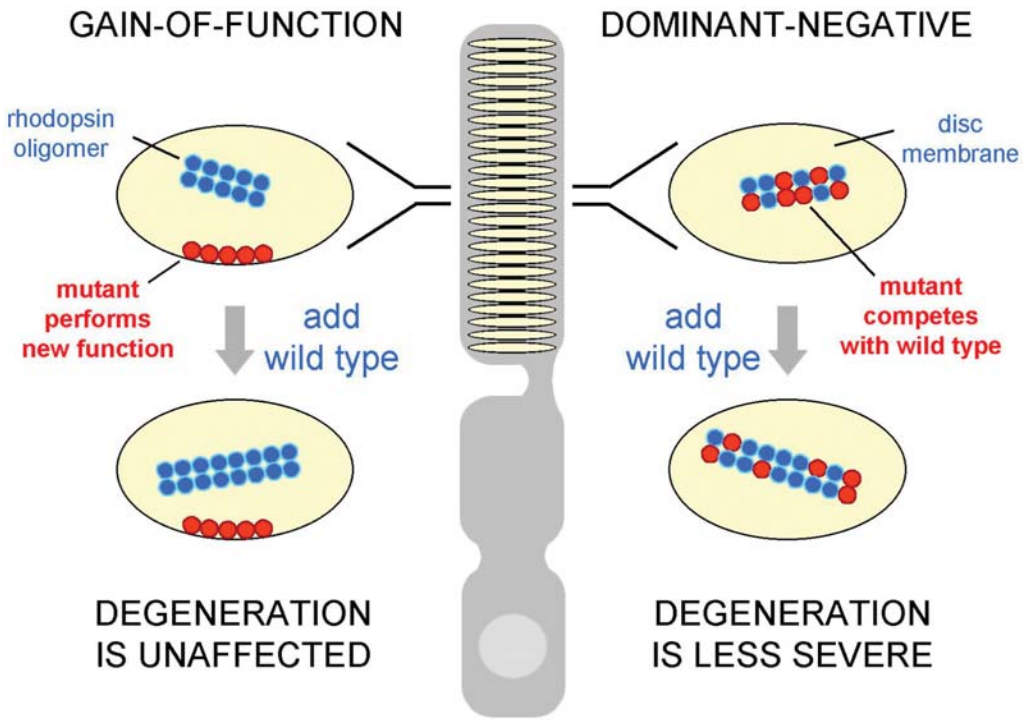

Fig. 1. Discrimination between gain-of-function and dominant-negative mutations. A section of disc membrane from a rod photoreceptor cell is enlarged to illustrate schematically the difference between gain-of-function and dominant-negative mutant rhodopsins. The novel function for the gain-of-function mutant is represented arbitrarily as alignment along the edge. Additional wild-type rhodopsin does not affect the new function, and retinal degeneration is unaffected. By contrast, dominant-negative rhodopsin mixes with wild-type rhodopsin. Additional wild-type dilutes the effect of the mutant rhodopsin, potentially rendering the degeneration less severe.

is the family of proteins to which rhodopsin belongs. If mutant forms of opsin preserved the ability to dimerize, they might interfere with normal function. When expressed in cultured cells, P23H displays an enhanced ability to oligomerize *(20).* Thus, the basic conditions are met for a dominant RP phenotype caused by dominant-negative mutations.

Discriminating Between Gain-of-Function and Dominant-Negative Mutations

In principle, gain-of-function or dominant-negative mutations in the rhodopsin gene could equally well account for the dominant RP phenotype. The classic way to discriminate between these two possibilities is to evaluate the phenotype under conditions in which the amount of normal protein is varied relative to the mutant protein (Fig. 1). Dominant-negative proteins compete with their normal counterparts, forming nonproductive complexes. This unproductive competition can be reduced by increasing the concentration of normal protein. By contrast, gain-of-function proteins, which have acquired some novel activity, are not in competition with the normal protein. Thus, changes in the concentration of the normal protein should not affect the severity of the dominant phenotype. In the absence of precise molecular information, this classic test provides

a useful practical definition of gain-of-function and dominant-negative mutations—one with critical relevance for gene therapy, as discussed here.

When this test was applied to a collection of dominant-negative *p53* mutants, the severity of the phenotype for more than half the mutants was significantly reduced in the presence of one additional copy of the wild-type gene *(26).* A different outcome was obtained for a gain-of-function mutation in the gene for superoxide dismutase (SOD1), which causes one type of amyotrophic lateral sclerosis *(30).* Mutant SOD1 accumulates in ubiquitinylated aggregates, and may share the same pathogenic mechanism suggested previously for the dominant P23H rhodopsin mutant *(22).* When mutant SOD1 was expressed as a transgene in mice that expressed 0%, 50%, 100%, or 700% of the normal amount of wild-type SOD1, the dominant phenotype was unchanged, consistent with the expectations for a gain-of-function mutation *(30).*

For rhodopsin mutants, this test is somewhat trickier because the health of the retina is sensitive to the level of expression of normal rhodopsin *(31,32).* Underexpression or overexpression of normal rhodopsin can cause retinal degeneration—the same phenotype as the dominant mutants. Therefore, the test must be performed in a range of expression that is compatible with a healthy retina. As shown in Table 1, mice that are heterozygous for null mutations have healthy retinas, establishing that one-half the normal amount of rhodopsin is adequate. Expression of transgenes for normal mouse or human rhodopsin, which appear to be equivalent, defines the upper limit as two- to threefold above normal (Table 1). Thus, in mice a dominant mutant (at a modest level of expression) can be tested on a background of wild-type rhodopsin that ranges between 50% and 200% of normal.

Several dominant mutants of human or mouse rhodopsin have been introduced as transgenes into mice (Table 1). At expression levels that would be compatible with a healthy retina, for wild-type rhodopsin, each of these mutants causes retinal degeneration, consistent with their phenotypes in humans. Only for the GHL transgene, which includes the P23H mutation, has expression been titrated against different levels of wild-type rhodopsin (*33,* Table 1). In that instance, the severe retinal degeneration observed in the presence of one normal allele was significantly reduced in the presence of a second copy of the wild-type gene (*33;* Table 1). Although not discussed by the authors, these results suggest that the GHL transgene behaves as if it carries a dominant-negative mutation, rather than a gain-of-function mutation.

The implication from mouse experiments that P23H is a dominant-negative mutation is at odds with results in cultured cells, which suggest that P23H is a toxic, gain-of-function mutation *(20).* The behavior of rhodopsin mutants in cultured cells may not mirror their behavior in the very different natural environment of the rod photoreceptor cell. The idea that the P23H mutation and other dominant rhodopsin mutations cause retinal degeneration by interfering with the proteasome system—toxic, gain of function—is very attractive because it links retinal degeneration to other neurodegenerative diseases through a common pathogenic mechanism *(20,22,23).* To resolve this issue, additional assays of retinal degeneration will be required for P23H and other rhodopsin mutations over the range of permissible rhodopsin concentrations. Such experiments could readily be done by breeding existing strains of mice (Table 1).

Implications for Gene Therapy of Dominant Rhodopsin Alleles

In contrast to a recessive disease, which can be treated by replacing the missing gene, dominant diseases present a more diverse set of conceptual and practical challenges *(2,3,9).* For dominant rhodopsin alleles, there are three general therapeutic strategies—silence the gene, knock it out, or fix it—that are independent of the nature of the mutation. These will work for gain-of-function and dominant-negative mutations alike. These strategies, which directly attack the offending gene or its RNA

product, have a nominal requirement to distinguish between the dominant allele and the normal copy of the gene, which generally differ by a single basepair. A ribozyme that is specific for the mRNA from a P23H allele has been shown to slow retinal degeneration in a rat model *(34,35).* For practical reasons, designing gene-therapy reagents for more than 100 different rhodopsin mutations is not economically viable: the cost of clinical validation would be prohibitive. This consideration has led to an alternative proposal: attack both genes simultaneously, and at the same time deliver a replacement copy that differs sufficiently in sequence so that it is not targeted by the therapeutic reagent *(9).* For example, one such reagent is an intron-directed triplex-forming oligonucleotide that would target both the wild-type and mutant genes, but bypass an intronless replacement construct *(36).* This approach offers the enormous practical advantage of allowing development of a single set of reagents that could then be used to treat all rhodopsin mutations.

In the absence of certain knowledge of the nature of the dominant mutations in the rhodopsin gene, mutation-independent strategies for gene therapy offer the surest approach. Lumping gain-of-function and dominant-negative mutations together, however, obscures the simpler form of gene therapy available for dominant-negative mutations—namely, supplementation with additional wild-type copies. If most rhodopsin mutations prove to be dominant-negatives, as suggested by the results with the GHL transgene in Table 1, supplementation would offer a general, economically valid approach. In contrast to treatment of recessive diseases, in which the addition of a wild-type gene is curative, treatment of dominant-negatives would be expected to reduce the severity of the retinal degeneration to an extent that depends on the effectiveness of the competition between the wild-type and mutant rhodopsin. To be useful, such a therapy must be effective within a range of overall rhodopsin concentration that is compatible with a healthy retina. The susceptibility of individual mutations to such therapy could be worked out in mice as a prelude to trials with large animals and nonhuman primates.

Although a cure is obviously desirable, treatments of RP need only slow the course of retinal degeneration to help patients. It seems likely that successful therapy will involve a combination of approaches that are now being attempted, and ones that have yet to be conceived *(2,3).* Testing the possible combinations of potential therapies challenges the efficiency of testing in existing animal models. An evaluation of treatments in living animals would be especially useful. Molecules such as the GFP-tagged rhodopsin in Table 1 (36; F. Chan, T.G. Wensel, and J.H. Wilson, unpublished results) would allow individual cellular components of the retina to be viewed *in situ.* In combination with adaptive optics *(37,38)* such visible tracers might allow for rapid assessment of treatment options in living animals.

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