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Rpe65 as a modifier gene for inherited retinal degeneration

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Abstract

Light accelerates progression of retinal degeneration in many animal models of retinitis pigmentosa (RP). A sequence variant in the *Rpe65* gene (*Rpe65*_{450Leu} or *Rpe65*_{450Met}) can act as a modulator of light-damage susceptibility in mice by influencing the kinetics of rhodopsin regeneration and thus by modulating the photon absorption. Depending on exposure duration and light intensity applied, white fluorescent light induces photoreceptor apoptosis and retinal degeneration in wild-type mice by the activation of one of two known molecular pathways. These pathways depend, respectively, on activation of the transcription factor c-Fos/AP-1 and on phototransduction activity. Here we tested *Rpe65* as a genetic modifier for inherited retinal degeneration and analysed which degenerative pathway is activated in a transgenic mouse model of autosomal dominant RP. We show that retinal degeneration was reduced in mice expressing the *Rpe65*_{450Met} variant and that these mice retained more visual pigment rhodopsin than did transgenic mice expressing the *Rpe65*_{450Leu} variant. In addition, lack of phototransduction slowed retinal degeneration whereas ablation of c-Fos had no effect. We conclude that sequence variations in the *Rpe65* gene can act as genetic modifiers in inherited retinal degeneration, presumably by regulating the daily rate of photon absorption through the modulation of rhodopsin regeneration kinetics. Increased absorption of photons and/or light sensitivity appear to accelerate retinal degeneration via an apoptotic cascade which involves phototransduction but not c-Fos.

Keywords

c-Fos/AP-1; mouse; phototransduction; retinal degeneration; Rpe65

Introduction

Age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are leading causes of blindness in the Western world. Whereas AMD is a multifactorial disease including exogenous (smoking, light exposure, etc.) and endogenous (gene mutation) risk factors, RP is mostly monogenic. Mutations in genes encoding proteins involved in visual function are especially prone to cause retinal degeneration. One of the most prominent mutations is found at position 23 of rhodopsin. The exchange of proline with histidine at this position (P23H) is responsible for ~10% of autosomal dominant RP in the USA.

The molecular events during retinal degenerations remain largely unknown although it would be essential to understand the mechanisms of the disease process to develop therapeutic strategies for patients. Light exposure accelerates the disease process in several animal models

and it has been suggested that light is a cofactor for AMD as well as for inherited retinal degenerations in human patients (Taylor *et al.*, 1990; Cruickshanks *et al.*, 1993; Simons, 1993; Cideciyan *et al.*, 1998; Cruickshanks *et al.*, 2001). Therefore, light-induced retinal degeneration in wild-type mice has been used to study pathophysiological mechanisms in the retina (Sanyal & Hawkins, 1986; Wang *et al.*, 1997; Chen *et al.*, 1999a; Chen *et al.*, 1999b; LaVail *et al.*, 1999; Organisciak *et al.*, 1999; Wenzel *et al.*, 2005a), and at least two different pathways for light-dependent photoreceptor cell death have been revealed. The first pathway, induced by short-term exposure to high light levels, depends on the activation of the transcription factor activator protein-1 (AP-1) but is independent of phototransduction. The second pathway, induced by long-term exposure to low levels of light, does not seem to depend on AP-1 but involves phototransduction (Hao *et al.*, 2002). It has been shown that a sequence variation in the *Rpe65* gene is associated with reduced light-damage susceptibility (LaVail *et al.*, 1987a,b; Danciger *et al.*, 2000). This variant codes for methionine instead of leucine at position 450 of the protein (RPE65_{450Met}) causing reduced steady-state levels of RPE65, probably via a post-translational mechanism (Wenzel *et al.*, 2001). The reduced RPE65 ('RPE' signifies 'retinal pigment epithelium') levels lead to a reduced rhodopsin regeneration rate in darkness (Wenzel *et al.*, 2001) and during illumination (Wenzel *et al.*, 2005b).

The VPP mouse is a model for autosomal dominant RP caused by the P23H mutation. It carries a rhodopsin transgene coding for a protein with three amino acid substitutions (V20G, P23H and P27L), one of which represents the P23H mutation in humans (Naash *et al.*, 1996). Retinal degeneration in mice expressing the *VPP* transgene is accelerated by light (Wang *et al.*, 1997). Genetic factors modulating disease progression have been postulated (Naash *et al.*, 1996).

Here we used the VPP mouse to study factors known to modulate light-damage susceptibility in a model of inherited retinal degeneration. By creating several double-mutant mice, we show that *Rpe65* acts as a modifier gene for retinal degeneration and that *VPP*-induced photoreceptor death follows the pathway of low-level long-term light exposure.

Materials and methods

Animals

All procedures concerning animals were in accordance with the regulations of the Veterinary Authority of Zurich and with the ARVO statement for the use of animals in research. Animals were kept in a 12:12-h light: dark cycle with 40–60 lux of dim white light within cages. To grow animals in constant darkness, animals were put into a wooden box at birth and kept in darkness for 6 weeks. Husbandry was carried out in dim red light with a wavelength > 650 nm. All mice used were of a mixed 129SV/C57BL/6 background. Double-transgenic mice were generated by classical breeding schemes. Genotypes were tested by polymerase chain reaction (PCR) of DNA isolated from tail tissue.

Assessment of retinal degeneration

Mice were killed with CO₂ and retinal degeneration was assessed qualitatively using light microscopy on 0.5- μ m sections of Epon-embedded tissues. Sections shown are from the lower temporal part of the retina. Quantitative determination of photoreceptor degeneration was carried out by measuring rhodopsin content as described previously (Kueng-Hitz *et al.*, 2000). Statistical analyses used unpaired two-tailed *t*-tests.

RNA isolation and RT-PCR

Total retinal RNA and cDNA were prepared using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) as described (Grimm *et al.*, 2000a). cDNA was prepared using oligo(dT)

and M-MLV reverse transcriptase (Promega, Madison, USA). cDNAs corresponding to 10 ng of total RNA were amplified for 23 cycles with primers specific for β -actin (up, 5'-CAA CGG CTC CGG CAT GTG C-3'; down, 5'-CTC TTG CTC TGG GCC TCG-3') or for 25 cycles with primers specific for *c-fos* (up, 5'-CAA CGC CGA CTA CGA GGC GTC AT-3'; down, 5'-GTG GAG ATG GCT GTC ACC G-3'), respectively (Grimm *et al.*, 2000a). Products were separated on a 1.5% agarose gel and visualized with ethidiumbromide staining.

Results

Rpe65 is a modifier gene for inherited retinal degeneration

We combined the *VPP* transgene with each of the *Rpe65* variants (*Rpe65*_{450Leu} and *Rpe65*_{450Met}) and tested the time course of retinal degeneration qualitatively by microscopic analysis of retinal morphology and quantitatively by measuring rhodopsin content. Degeneration in both *VPP*;*Rpe65*_{450Leu} and *VPP*;*Rpe65*_{450Met} mice was not uniform; this is manifested in an increased severity of photoreceptor loss in the temporal retina as compared to the nasal retina (not shown). Micrographs shown in Fig. 1 (and in all other figures) were taken from the most affected region. Independent of the *Rpe65* variant, *VPP* and wild-type mice showed a similar retinal morphology at postnatal day (PND) 11 (Fig. 1A, top panels). At PND 15, thinning of the outer nuclear layer (ONL) was observed in all *VPP* mice but mice with the *Rpe65*_{450Leu} variant appeared to be more severely affected. At PND 18, thinning of the ONL had continued and photoreceptor outer segments were much shorter than in wild-type mice (Fig. 1A, bottom panels). At PND 21, when developmental apoptosis is completed, the number of photoreceptors in *VPP* mice was reduced, with the *Rpe65*_{450Leu} variant slightly more affected (Fig. 1B, top panels). Photoreceptor outer segments were poorly preserved. From PND 21 to PND 56, progression of the degeneration was slow or almost nonexistent. Indeed, retinal morphology seemed to improve in the *VPP*;*Rpe65*_{450Met} mouse strain up to PND 42. However, such a potential improvement was not represented by a statistically significant increase in the content of total retinal rhodopsin ($P = 0.098$, PND 21 vs. PND 42). Furthermore, expression studies of the proliferation marker ki-67 by reverse transcription (RT)-PCR and immunohistochemical detection assays of systemically applied BrdU did not show evidence of proliferative activity in the ONL of mice of the *VPP*;*Rpe65*_{450Met} genotype (not shown). A similar biphasic degeneration as observed here (a fast phase until PND 21 and slow phase thereafter) was recently described for transgenic rats homozygous for the P23H mutation (Pinilla *et al.*, 2005).

Nevertheless, at PND 28 ($P = 0.0046$) and PND 42 ($P < 0.0001$), retinas of *VPP* mice expressing the *RPE65*_{450Met} variant had significantly higher rhodopsin values than retinas of *VPP* mice expressing the *RPE65*_{450Leu} variant (Fig. 1C). This suggests that the two *Rpe65* sequence variants have different influences on the morphology and rhodopsin content of the retinas in the degenerative process caused by the *VPP* transgene.

Complete removal of *RPE65* by crossing the *VPP* transgene onto an *Rpe65*-knockout background slightly delayed the degeneration but did not protect the retina to the same extent as the *Rpe65*_{450Met} variant. Of note, deletion of *Rpe65* alone causes a late onset and slowly progressing retinal degeneration (Rohrer *et al.*, 2003; Woodruff *et al.*, 2003), which proceeds much more slowly than observed here for *Rpe65*-deficient *VPP* mice (Fig. 1B and D).

The progression of *VPP*-mediated retinal degeneration is not only dependent on the genetic background but is also accelerated by light (Naash *et al.*, 1996; Wang *et al.*, 1997). The two variants of the *Rpe65* gene influence rhodopsin regeneration in the visual cycle (Wenzel *et al.*, 2001) and therefore modulate the photon absorption capacity by the visual pigment during light exposure (Wenzel *et al.*, 2005b). To eliminate the influence of light, *VPP*;*Rpe65*_{450Leu} mice were raised in darkness. This treatment partly protected retinal morphology at PND 42

as compared to animals of the same genotype reared in cyclic light (Fig. 2A). The morphological observation was supported by rhodopsin values which were approximately two-fold higher when the animals were reared in darkness ($P = 0.0164$). In fact, dark-rearing of *VPP;Rpe65^{450Leu}* mice resulted in rhodopsin levels that were no longer statistically significantly different ($P = 0.304$) from those of mice with the *VPP;Rpe65^{450Met}* genotype reared in cyclic light (Fig. 2B).

Retinal degeneration in the VPP mice involves phototransduction

Depending on the intensity and duration, respectively, of exposure, light-induced retinal degeneration depends on phototransduction and activation of c-Fos/AP-1 (Hao *et al.*, 2002). To test the potential involvement of c-Fos/AP-1 in the degenerative process in the VPP mice, we determined the relative expression levels of *c-fos* mRNA by RT-PCR during postnatal development and degeneration in retinas of wild-type and transgenic animals. The *c-fos* mRNA expression showed a similar initial increase in both strains of mice and reached high levels especially in retinas of adult VPP mice during the degenerative period (Fig. 3A). Although mRNA expression may not always reflect protein levels, this result indicates a potential role for c-Fos in retinal degeneration in the VPP mouse. To test this hypothesis, we crossed the *VPP* transgene onto the *c-fos*-null background. As judged by morphological appearance at PND 28, lack of c-Fos did not rescue retinal degeneration in the VPP mouse (Fig. 3B). When the global degeneration was assessed by the measurement of rhodopsin in whole retinas, the two types of mice had similar levels of the visual pigment. In wild-type mice, lack of c-Fos leads to a reduction in the rhodopsin content by 20% (Kueng-Hitz *et al.*, 2000). A similar reduction in retinal rhodopsin levels was evident when VPP mice were compared to *VPP;c-fos^{-/-}* mice (Fig. 3C).

To test the potential involvement of phototransduction in the degenerative process, we ablated transducin by combining the *VPP* transgene with the guanine nucleotide-binding protein G(t), alpha-1 subunit, gene knockout (*Gnat1a^{-/-}*). This genetic block of rod phototransduction resulted in protection of photoreceptors at least up to PND 42 (Fig. 4A). In addition, photoreceptor outer segments were much longer but preservation of the outer segment structure was poor. Outer segments showed large vesiculations and condensations. Nevertheless, retinas from *VPP;Gnat1a^{-/-}* mice had significantly higher rhodopsin contents than VPP mice at PND 28 ($P = 0.0166$) and at PND 42 ($P = 0.0053$; Fig. 4B).

Discussion

Exposure to light above a given threshold induces retinal degeneration in wild-type animals. Similarly, environmental light accelerates photoreceptor apoptosis in many models of inherited retinal degeneration. Here we show that these events share some common molecular mechanisms. The degree of damage or the rate of disease progression can be modulated by regulating the availability of rhodopsin and the activity of the phototransduction cascade. It has been suggested that the genetic background influences progression of inherited retinal degeneration. We have identified RPE65 as one of the proposed genetic modifiers. Our findings indicate that sequence variants in RPE65 differentially influence progression of inherited retinal degeneration through the modulation of light sensitivity (the rate of photon absorption) and the subsequent initiation of phototransduction by activated rhodopsin.

RPE65 is the isomerohydrolase essential for the generation of 11-*cis*-retinal in the visual cycle (Jin *et al.*, 2005; Moiseyev *et al.*, 2005; Redmond *et al.*, 2005). Lack of functional RPE65 results in the almost complete absence of rhodopsin and the accumulation of retinylester in the RPE (Redmond *et al.*, 1998; Seeliger *et al.*, 2001; Gollapalli *et al.*, 2003). The recently identified RPE65 variant 450Met causes a 13-fold reduction in the steady-state level of RPE65 protein in the RPE (Lyubarsky *et al.*, 2005), presumably by a post-translational process

(Wenzel *et al.*, 2001). This reduces rhodopsin regeneration kinetics after bleaching more than three-fold (Lyubarsky *et al.*, 2005). As rhodopsin is essential for light damage (Grimm *et al.*, 2000b), pharmacological (Keller *et al.*, 2001; Sieving *et al.*, 2001) or genetic (Wenzel *et al.*, 2001) interference with efficient rhodopsin regeneration leads to a reduced light-damage sensitivity. Similarly, introducing the *Rpe65*_{450Met} variant attenuated progression of photoreceptor cell loss in the inherited retinal degeneration mediated by the *VPP* transgene. This indicates that the *Rpe65* gene can act as a genetic modifier for inherited retinal degenerations, presumably through the modulation of the rate of photon absorption by rhodopsin.

Naash and coworkers showed that *VPP*-mediated degeneration depends cumulatively on the genetic background and light exposure (Naash *et al.*, 1996). The influence of the genetic background was tested by comparing *VPP*-induced retinal degeneration in albino and pigmented mice. The *Rpe65*_{450Leu} variant is found in most mouse strains including FVB (M. Danciger, personal communication) from which the albino mice used in the Naash studies were derived. It is therefore probable that the albino *VPP* mice carried the *Rpe65*_{450Leu} variant and that the pigmented animals (which were derived from the *Rpe65*_{450Met}-expressing C57BL/6 strain) used for comparison carried the *Rpe65*_{450Met} variant. When Naash and coworkers raised albino and pigmented *VPP* mice in cyclic light, the rhodopsin content in pigmented animals was roughly three times as much as in the albino *VPP* mice. We found a very similar difference between *VPP;Rpe65*_{450Leu} and *VPP;Rpe65*_{450Met} mice at PND 42 (Fig. 1). Furthermore, the increase in rhodopsin content in the *VPP;Rpe65*_{450Leu} mouse after dark-rearing closely resembles the increase in the dark-reared albino *VPP* mice reported by Naash and colleagues. This suggests that the genetic component Naash *et al.* (1996) proposed may be found in the *Rpe65* gene. It was also reported that the sequence variants of *Rpe65* can influence disease progression in a model of Stargardt disease: in the *ABCR*^{-/-} mouse, the *Rpe65*_{450Met} variant decreased accumulation of A2E in the RPE, presumably by modulating photon absorption and rhodopsin bleaching (Kim *et al.*, 2004).

However, the difference in photon absorption capacity between mice with the methionine and the leucine variants may not fully account for the different degeneration rates observed in the different *VPP* mice. Otherwise, dark-reared *VPP;Rpe65*_{450Leu} mice should show higher rhodopsin levels than *VPP;Rpe65*_{450Met} mice reared in cyclic light. Thus, we postulate additional genetic factor(s) which may cosegregate with *Rpe65*. New quantitative trait loci influencing retinal damage in models of light- and age-related degeneration have recently been identified on several mouse chromosomes. However, none of these loci map to distal chromosome 3, the genetic location of *Rpe65* (Danciger *et al.*, 2003; Danciger *et al.*, 2004; Danciger *et al.*, 2005). It will nevertheless be of importance to identify these factors and test them in combination with models of inherited retinal degeneration.

Interestingly, complete absence of RPE65 function, a condition dramatically reducing the photon catch capacity of the retina (Seeliger *et al.*, 2001), did not notably reduce the rate of retinal degeneration in *VPP* mice. Lack of RPE65 on its own causes an early cone degeneration (Znoiko *et al.*, 2005) followed by a slowly progressing rod cell death. Thus, by combining the *VPP* transgene with a *Rpe65* deletion, two effects may sum and cause the degeneration observed. An alternative explanation is provided by recent findings made in cell-based or purified *in vitro* systems: Noorwez *et al.* (2004) found that opsin with the P23H mutation incorporated 11-*cis*-retinal more efficiently when the chromophore was present during synthesis of the protein. Complexation of P23H opsin with 11-*cis*-retinal stabilized the mutant rhodopsin facilitating its cellular folding and transport (Noorwez *et al.*, 2004). In the absence of RPE65 protein and therefore of a functional visual cycle, no or only minimal amounts of 11-*cis*-retinal is present to assist biogenesis of mutant rhodopsin. This may lead to an increased abundance of un- or misfolded and/or of mislocalized proteins. As a consequence, large

aggregates of mutant opsin may form and impair the ubiquitin proteasome system, resulting in cytotoxic effects and cell death (Illing *et al.*, 2002; Saliba *et al.*, 2002). Accumulation of high amounts of unfolded proteins may additionally elicit a cytotoxic effect by affecting the unfolded protein response (Bucciantini *et al.*, 2002; Forman *et al.*, 2003; Forman *et al.*, 2004). Intriguingly, such mechanisms can also be found in other neurodegenerative diseases such as Alzheimer's disease. An additional explanation for the lack of rescue in the *VPP;Rpe65^{-/-}* mice argues for increased signalling of unliganded opsin. It has been shown that the degeneration observed in the *Rp65^{-/-}* mouse retina can be blocked by the genetic ablation of phototransduction (Woodruff *et al.*, 2003). To understand the pathophysiological signals leading to photoreceptor cell death in inherited retinal degenerations, it will be of crucial importance to elucidate the molecular mechanisms of such light-independent degenerative processes.

At least two different apoptotic pathways downstream of photon absorption and rhodopsin activation exist and these mediate light-induced photoreceptor degeneration in wild-type mice: short exposure to high-level light depends on c-Fos/AP-1 and is independent of transducin (and therefore phototransduction), while long exposure to low-level light is independent of c-Fos and dependent on transducin (Hao *et al.*, 2002). To discriminate between these two pathways for the deleterious effect of light in the VPP mouse, we analysed retinal degeneration in VPP mice deficient for c-Fos and VPP mice deficient for transducin, respectively. Although *c-fos* gene expression was strongly induced in VPP mice (Fig. 4A), ablation of c-Fos did not alter the degenerative process (Fig. 4B and C). In contrast, lack of transducin considerably preserved the number of photoreceptor nuclei. This strongly suggests that VPP-mediated retinal degeneration follows a pathway similar to that induced in wild-type mice by long-term low-level light exposure.

Collectively, our data reinforce the observation that progression of RP can be enhanced by light acting directly on the diseased photoreceptor. Thus, reducing light exposure is advisable for patients suffering from RP. However, it is similarly important to identify and understand the molecular mechanisms enforced by modifier genes. For a successful pharmacological intervention in the disease process, it thus may be necessary not only to target the consequences of the primary genetic defect but also to pay close attention to the function of potential modifier genes.

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Abbreviations

AMD	age-related macular degeneration
AP-1	activator protein-1
ONL	outer nuclear layer
PCR	polymerase chain reaction
PND	postnatal day
RP	retinitis pigmentosa
RPE	retinal pigment epithelium

RT reverse transcription

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Fig. 1.

Time course of VPP-mediated retinal degeneration was dependent on the *Rpe65* sequence. (A) Retinal morphology of VPP;*Rpe65*_{450Met} (left column), VPP;*Rpe65*_{450Leu} (middle column) and wt;*Rpe65*_{450Leu} (right column) mice at PND 11 (top row), 15 (middle row) and 18 (bottom row). Shown are representative sections of the lower temporal part of at three or more independent mice. (B) Retinal morphology of VPP;*Rpe65*_{450Met} (left column), VPP;*Rpe65*_{450Leu} (middle column) and *Rpe65*^{-/-} (right column) mice at PND 21 (top row), 28 (second row), 42 (third row) and 56 (bottom row). The *Rpe65*_{450Leu} variant accelerated the VPP-mediated retinal degeneration leading to a more pronounced thinning of the ONL and less preserved photoreceptor segments. The *Rpe65* knockout only partially rescued the degeneration. Shown are representative sections of the lower temporal part of at least three

independent mice. (C) Rhodopsin per retina expressed in nmol. Values are given as means + SD. Open bars, *VPP;Rpe65^{450Met}*; solid bars, *VPP;Rpe65^{450Leu}*. Numbers of mice are indicated. **P* = 0.0046, ***P* < 0.0001. (D) Representative section through the lower temporal part of the retina of an *Rpe65^{-/-}* mouse at PND 56. PS, photoreceptor segment; ONL, outer nuclear layer; INL, inner nuclear layer.

Fig. 2.

Dark rearing partially rescued retinal degeneration in *VPP;RPE65_{450Leu}* mice. (A) Representative sections of the lower temporal retina of mice reared in cyclic light (left) or reared in darkness (right) for 42 days. Mice were of the *Rpe65_{450Leu}* genotype. (B) Rhodopsin per retina [nmol] in *VPP;Rpe65_{450Leu}* and *VPP;RPE65_{450Met}* mice reared in cyclic light and in dark-reared *VPP;Rpe65_{450Leu}* mice as indicated. Values are given as means + SD. Numbers of retinas are indicated. * $P = 0.0164$. Rhodopsin values in dark-reared *VPP;Rpe65_{450Leu}* mice were not significantly different ($P = 0.304$) from rhodopsin values in cyclic-reared *VPP;RPE65_{450Met}* mice.

Fig. 3.

VPP-mediated retinal degeneration was independent of c-Fos. (A) *c-fos* gene expression in retinas isolated from wt or VPP mice at the PNDs indicated. Total RNA from three retinas of three mice were pooled for RT-PCR analysis at each timepoint; M, 100-bp DNA marker. (B) Representative sections of the lower temporal retina of PND 28 VPP or VPP;*c-fos*^{-/-} mice as indicated. All mice were of the *Rpe65*^{450Met} genotype. (C) Rhodopsin per retina expressed in nmol. Values are given as means + SD. Numbers of retinas are indicated. **P* < 0.0001.

Fig. 4.

VPP-mediated retinal degeneration was influenced by phototransduction. (A) Representative sections of the lower temporal retina of *VPP* and of *VPP;Gnat*^{-/-} mice raised in cyclic light. Lack of functional rod transducin improved retinal morphology in *VPP;Rpe65*^{450Leu} mice: an increased number of photoreceptor nuclei in the ONL is apparent and photoreceptor segments are better preserved. All mice were of the *Rpe65*^{450Leu} genotype and were taken at the PNDs indicated. (B) Rhodopsin per retina expressed in nmol. Values are given as means + SD. Solid bars, *VPP;Rpe65*^{450Leu}; open bars, *VPP;Gnat*^{-/-}; *Rpe65*^{450Met}. Numbers of retinas are indicated. **P* = 0.0166, ***P* = 0.0053. Abbreviations as in Fig. 1.