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Atypical retinal degeneration 3 in mice is caused by defective PDE6B pre-mRNA splicing ☆

Hakim Muradov, Kimberly K. Boyd, Vasily Kerov, Nikolai O. Artemyev *

Department of Molecular Physiology and Biophysics, University of Iowa Carver College of Medicine, Iowa City, IA 52242, United States

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ABSTRACT

Mutations in the key rod phototransduction enzyme phosphodiesterase 6 (PDE6) are known to cause recessive retinitis pigmentosa in humans. Mouse models of mutant PDE6 represent a common approach to understanding the mechanisms of visual disorders related to PDE6 defects. Mutation N605S in the PDE6B subunit is linked to atypical retinal degeneration 3 (*atrd3*) in mice. We examined PDE6 in *atrd3* mice and an *atrd3* mutant counterpart of human cone PDE6C expressed in rods of transgenic *Xenopus laevis*. These animal models revealed remarkably different phenotypes. In contrast to dramatic downregulation of the mutant rod PDE6 protein and activity levels in mice, expression and localization of the cone PDE6C in *X. laevis* were essentially unaffected by this mutation. Examination of the PDE6B mRNA in *atrd3* retina showed that the mutation-carrying exon 14 was spliced-out in the majority of the transcript. Thus, retinal degeneration in *atrd3* mice is caused by low levels of PDE6 protein due to defective processing of PDE6B pre-mRNA rather than by deleterious effects of the N605S mutation on PDE6 folding, stability or function.

1. Introduction

Rod and cone photoreceptor cells respond to light stimuli by activating phosphodiesterase-6 (PDE6) that hydrolyze cGMP thereby modulating cGMP-gated channels in the plasma membrane (Burns & Arshavsky, 2005; Fu & Yau, 2007; Lamb & Pugh, 2006). Rod PDE6 is a heterotetramer composed of two catalytic subunits, PDE6A and PDE6B, and two copies of the small inhibitory Pγ subunit. Cone PDE6 is highly homologous to the rod enzyme and is a tetramer comprised of two identical catalytic subunits PDE6C and two copies of a cone-specific Pγ subunit (reviewed in Zhang and Cote (2005)). Mutations in the *PDE6A* and *PDE6B* genes are responsible for a significant fraction of cases of recessive retinitis pigmentosa (RP), a common disease of progressive photoreceptor degeneration leading to loss of vision. In addition, mutations in rod PDE6 may lead to a non-progressive night blindness (Dryja et al., 1999; Huang et al., 1995; McLaughlin et al., 1993). The PDE6B H258N mutation was reported in the Rambusch form of dominant stationary night blindness (Gal et al., 1994). Recently, *PDE6C* mutations have been found in human patients with progressive cone dystrophy and achromatopsia (Chang et al., 2009; Thiadens et al., 2009). These disorders of cone function are characterized by low visual acuity and lack of color discrimination. Certain pathogenic PDE6 alterations such as nonsense mutations, frame-shifts, or splicing defects apparently result in complete loss of PDE6 function. Often PDE6 mutations in RP and achromatopsia are missense mutations leading to substitutions of conserved residues within the regulatory GAF domains or the catalytic domains. The mechanisms of such PDE6 mutations are not understood and may include loss of PDE6 function due to protein folding defects, deficient transport of PDE6 to the site of phototransduction, or abnormal function of the enzyme.

Despite numerous efforts, heterologous expression of PDE6 has not been attained. Thus, characterization of animal models has been the main approach to understanding the mechanisms of human visual disorders caused by mutations of the PDE6 catalytic subunits. Mouse models with spontaneous or chemically-induced mutations in PDE6B and, more recently, PDE6A and PDE6C have been identified (Bowes et al., 1990; Chang et al., 2007, 2009; Hart et al., 2005; Sakamoto et al., 2009; Pittler & Baehr, 1991; Thaung et al., 2002). The *rd1* mouse is a classical loss-of-function model with disruption of the *PDE6B* gene causing elevation of cGMP levels and rapid retinal degeneration (Bowes et al., 1990; Farber & Lolley, 1974; Pittler & Baehr, 1991). Similarly, a spontaneous disruption of the *PDE6C* gene in the *cpfl1* mutant mouse causes rapid degeneration of cones (Chang et al., 2009). However, mouse models with

Abbreviations: PDE6, cone or rod outer segment cGMP phosphodiesterases; Pγ, inhibitory subunit of PDE6; ONL, outer nuclear layer; OS/IS, outer segment(s)/inner segment(s) of photoreceptor cells; *atrd3*, atypical retinal degeneration 3.

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* Corresponding author. Fax: +1 319 335 7330.
E-mail addresses: Hakim-Muradov@uiowa.edu (H. Muradov), Kimberly-Boyd@uiowa.edu (K.K. Boyd), bazilione@gmail.com (V. Kerov), nikolai-artemyev@uiowa.edu (N.O. Artemyev).

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missense mutations in rod PDE6 are more representative of the majority of PDE6-related cases of RP. The progression of photoreceptor degeneration in these animal models is slower than in *rd1* mice, theoretically allowing examination of biochemical outcomes of PDE6 mutations. Furthermore, a slow progression of retinal degeneration in mutant mice potentially indicates change of function as opposed to loss-of-function mutations.

Three recessive *PDE6B* alleles causing a relatively slow onset of retinal degeneration were originally selected in a screen for novel N-ethyl-N-nitrosourea-induced mutations that lead to vision disorders in the mouse (Hart et al., 2005; Thaung et al., 2002). The mutant alleles were termed *atrd1–atrd3* for atypical retinal degeneration (Thaung et al., 2002). The *atrd3* allele appeared particularly interesting (Barren et al., 2009; Hart et al., 2005). This allele was originally reported to carry the N606S mutation (Hart et al., 2005). However, our sequencing of genomic DNA obtained from *atrd3* mice indicated a N605S mutation, which is in agreement with the current annotation of the mutant allele at the Mouse Genome Informatics database (ID: MGI: 2178316). The position corresponding to Asn^{605} in mouse PDE6B is not absolutely conserved among PDEs. PDE1 and PDE3 contain a Thr residue, whereas PDE4 and PDE10 contain a Ser residue at this position, which is analogous to the Asn → Ser substitution in *atrd3* mice. Moreover, the atomic structure of the chimeric PDE5/PDE6 catalytic domain complexed with the inhibitory peptide of Pγ revealed that the Asn residue contributes to the Pγ-binding site, suggesting a potential functional defect of the *atrd3* mutant (Barren et al., 2009). To investigate the mechanism of the PDE6B N605S mutation in retinal degeneration, we examined PDE6 in *atrd3* mice. In addition, we generated transgenic *X. laevis* expressing an *atrd3* counterpart of EGFP-fused human PDE6C in rods, PDE6C-N610S. Surprisingly, the two animal models revealed strikingly different phenotypes. In mouse *atrd3* retina, levels of PDE6B expression and total PDE6 activity were markedly downregulated, whereas in frog retina, expression and localization of EGFP-PDE6C were essentially unaffected by this mutation. The difference in the phenotypes is reconciled by the finding of the abnormal PDE6B pre-mRNA splicing in mutant mice leading to very low expression levels of PDE6.

2. Methods

2.1. Animals

Homozygous $atrd3^{+/+}$ female mice were obtained from the Mary Lyon Center (England). The mice were mated with wild-type mice (C57Bl/6) to obtain heterozygous $atrd3^{+/-}$ mice, which were subsequently bred to obtain homozygous $atrd3^{+/+}$ mice. Mice were genotyped by PCR-amplification and DNA sequencing of the mutant *PDE6B* region. Transgenic *X. laevis* expressing EGFP-PDE6C in rods were generated by the method of sperm nuclear transplantation as previously described (Muradov et al., 2009). The N610S mutation was introduced by PCR-directed mutagenesis into the PDE6C DNA sequence which was then cloned into the pXOP(−508/+41)EGFP vector using NotI and XmaI sites. The construct sequence was confirmed by automated DNA sequencing. *X. laevis* tadpoles expressing EGFP-PDE6C-N610S were obtained similarly as described for EGFP-PDE6C (Muradov et al., 2009). All experimental procedures involving the use of mice and frogs were carried out in accordance with the protocol approved by the University of Iowa Animal Care and Use Committee.

2.2. Immunofluorescence

Mouse eyeballs were enucleated and the corneas were perforated with a 21 gauge needle, fixed in PBS with 4% formaldehyde for 1 h and cut in half. The cornea and lens were removed, and the eyecups were submersed in a 30% sucrose solution in PBS for 5 h at 4 °C. The eyecups were then embedded in tissue freezing medium (Triangle Biomedical Sciences) and frozen on dry ice. Radial sectioning (10 μm) of the retina was performed using a cryomicrotome HM 505E. Retinal cryosections were air-dried and kept at −80 °C. The sections were warmed up to room temperature and incubated in PBS/0.1% Triton X-100 for 30 min. Labeling with anti-rhodopsin 1D4 monoclonal antibody (1:200) (Santa Cruz Biotech), rabbit anti-PDE6B PA1-722 (1:1000) (Thermo Scientific) antibody, anti-PDE6 MOE antibody (1:1000) (CytoSignal), or anti-Pγ-(63–87) antibody (1:1000; gift of Dr. R. Cote, University of New Hampshire) was performed in PBS containing 3% BSA for 1–2 h at 25 °C. Following 1-h incubation with goat anti-mouse AlexaFluor 488, goat anti-rabbit AlexaFluor 488, or goat anti-rabbit AlexaFluor 568 secondary antibodies (Invitrogen) (1:1000), the sections were visualized using a Zeiss LSM 510 confocal microscope. *In situ* cell death detection kit, TMR red (Roche), was used for TUNEL staining of retina cryosections according to manufacturer' protocol. The sections were counterstained with To-Pro3 (Invitrogen).

2.3. EGFP-fluorescence

At about stage 48–50, retinas from transgenic tadpoles were dissected into small pieces in a Ringer's buffer and the EGFP-fluorescence in living photoreceptor cells was imaged using LSM510 (Zeiss).

2.4. PDE6 extraction, immunoblotting, immunoprecipitation, and PDE6 activity assays

To determine the total levels of expression of PDE6 subunits, retinas of P10 $atrd3^{+/+}$ and wild-type (C57Bl/6) mice were homogenized in 10 mM Tris–HCl buffer pH 7.5 containing 1 mM 2-mercaptoethanol and 1% Triton X-100 (25 μl/retina) and centrifuged at 16,000*g* for 10 min. The supernatants were separated by SDS PAGE and analyzed by Western blotting with anti-PDE6 antibody 63F (gift of Dr. R. Cote, University of New Hampshire) and anti-PDE6B PA1-722 (Thermo Scientific). For PDE6 activity analyses, P10 $atrd3^{+/+}$ and WT retinas were homogenized with a pestle in an Eppendorf tube in 20 mM Tris–HCl buffer (pH 7.5) containing 120 mM NaCl, 1 mM $MgSO_4$, 1 mM 2-mercaptoethanol, and complete protease inhibitor cocktail (Roche) (30 μl/retina). Aliquots of retina homogenates were used for measurements of the total levels of PDE6 activity following trypsin-treatment (30 μg retina protein/2 μg trypsin, 0–20 min, 0 °C, terminated with 10× SBTI). 5-min treatment maximally activated PDE6 activity. The rest of the homogenates were centrifuged (20,000*g*, 20 min, 4 °C), and the resulting pellet was re-suspended in hypotonic 10 mM Tris–HCl (pH 7.5) buffer (15 or 30 μl/retina) containing 1 mM β-mercaptoethanol and complete protease inhibitor cocktail. The PDE6-containing hypotonic supernatants (70,000*g*, 60 min, 4 °C) were used immediately or stored at −80 °C. Samples of PDE6 extracts containing equal amounts of functional PDE6 were used for immunoprecipitation. Hypotonic PDE6 extract from 20 to 25 $atrd3^{+/+}$ retinas contained as much functional PDE6 as that from 1 WT retina as determined by PDE6 activity after trypsin-treatment. Dynabeads with Protein G (30 μl, 30 mg/ml) (Invitrogen) were washed with 20 mM Tris–HCl (pH 7.5) buffer containing 500 mM NaCl, 1 mM MgSO4, and 1 mM 2-mercaptoethanol (buffer A) and incubated with anti-PDE6B PA1-722 (5 μg) for 60 min at 25 °C. The beads were washed from unbound proteins two times with buffer A, followed by the addition of PDE6 extracts from WT, $Nrl^{-/-}$ or $atrd3^{+/+}$ retinas and incubation with rotation for 3 h at 4 °C. The beads were then washed four times with buffer A, one time with buffer A minus NaCl, and finally one time with buffer A. Resins