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Distinct patterns of compartmentalization and proteolytic stability of PDE6C mutants linked to achromatopsia

Pallavi Cheguru [a], Anurima Majumder [a], Nikolai O. Artemyev [a,b,*]

[a] Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA 52242, United States
[b] Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA 52242, United States

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ABSTRACT

Phosphodiesterase-6 (PDE6) is an essential effector enzyme in vertebrate photoreceptor cells. Mutations in rod and cone PDE6 cause recessive retinitis pigmentosa and achromatopsia, respectively. The mechanisms of missense PDE6 mutations underlying severe visual disorders are poorly understood. To probe these mechanisms, we expressed seven known missense mutants of cone PDE6C in rods of transgenic *Xenopus laevis* and examined their stability and compartmentalization. PDE6C proteins with mutations in the catalytic domain, H602L and E790K, displayed modestly reduced proteolytic stability, but they were properly targeted to the outer segment of photoreceptor cells. Mutations in the regulatory GAF domains, R104W, Y323N, and P391L led to a proteolytic degradation of the proteins involving a cleavage in the GAFb domain. Lastly, the R29W and M455V mutations residing outside the conserved PDE6 domains produced a pattern of subcellular compartmentalization different from that of PDE6C. Thus, our results suggest a spectrum of mechanisms of missense PDE6C mutations in achromatopsia including catalytic defects, protein mislocalization, or a specific sequence of proteolytic degradation.

1. Introduction

Cyclic-nucleotide phosphodiesterases (PDEs) of the sixth family (PDE6) are the key effector enzymes in phototransduction in rods and cones (Fu and Yau, 2007; Arshavsky and Burns, 2012). The enzyme catalytic core is a heterodimer of PDE6A and PDE6B subunits in rod PDE6 and a homodimer of PDE6C subunits in cone PDE6. Mutations in the *PDE6A* and *PDE6B* genes are responsible for a significant fraction of recessive retinitis pigmentosa (RP), an inherited degenerative retina disease leading to blindness (McLaughlin et al., 1995; Dryja et al., 1999). Mutations in *PDE6C* cause autosomal recessive achromatopsia (ACHM) (Chang et al., 2009; Thiadens et al., 2009; Grau et al., 2011). ACHM results from a loss of cone function and is characterized by low visual acuity and lack of color discrimination. Many mutations in PDE6, including nonsense mutations, splice defects, and frame shifts are certain to cause loss of expression, misfolding, and/or loss of PDE6 function. However, the mechanisms of missense PDE6 mutations in rods and cones leading to retina disease are largely unknown.

Missense mutations may alter PDE6 function or interfere with transport of functional PDE6 to the outer segment (OS), a specialized ciliary compartment of photoreceptor cells. Proper transport of PDE6 in photoreceptors is critically important for the function and survival of rods and cones. Lack of functional PDE6 in the rod OS leads to elevation of cGMP levels and causes rapid retinal degeneration (RD) in animal models and humans (Farber and Lolley, 1974; Bowes et al., 1990; Pittler and Baehr, 1991; Liu et al., 2004; Ramamurthy et al., 2004). The possibility that abnormal PDE6 trafficking may underlie RP is highlighted by the *PDE6B* mutation L854V in the protein C-terminal CAAX motif (Veske et al., 1995). Isoprenylation modifications of PDE6 are critical to the PDE6 interaction with membranes and transport (Anant et al., 1992; Catty et al., 1992; Veske et al., 1995; Karan et al., 2008). The C-terminal CAAX boxes of mammalian PDE6A and PDE6B specify farnesylation and geranylgeranylation, respectively (Anant et al., 1992). In an attempt to characterize PDE6C mutants linked to ACHM, several missense mutations were previously introduced into a chimeric PDE5/PDE6C enzyme expressed in sf9 cells (Grau et al., 2011). These mutants indicated either a loss or reduction of the catalytic activity (Grau et al., 2011). However, the use of PDE5/PDE6C chimeras to study the effects of PDE6 mutations has severe limitations. PDE5/PDE6C chimeras contained the catalytic domain of PDE5 and thus, they are essentially PDE5-like enzymes. Furthermore, the process of PDE6 folding, assembly, and trafficking in rod and cones is complex and it involves photoreceptor-specific protein machinery (Ramamurthy et al., 2004; Karan et al., 2008). These aspects cannot be recapitulated using the chimera/insect cell system.

Abbreviations: PDE6, photoreceptor phosphodiesterase-6; PDE5, cGMP-binding, cGMP-specific phosphodiesterase-5; ACHM, autosomal recessive achromatopsia; GAF, domains, termed for their presence in cGMP-regulated PDEs, *a*denylyl cyclases, and the *E. coli* protein *F*h1A; OS, outer segment; IS, inner segment; WGA, Wheat Germ Agglutinin; DTT, dithiothreitol.

* Corresponding author at: Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA 52242, United States.
E-mail address: nikolai-artemyev@uiowa.edu (N.O. Artemyev).

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Our previous studies demonstrated that transgenic *Xenopus laevis* is a robust system to investigate PDE6 and its transport (Muradov et al., 2009, 2010). Human EGFP-tagged PDE6C ectopically expressed in rods of transgenic *X. laevis* traffics correctly to the OS. In the OS, EGFP-PDE6C concentrates at the disc rims and co-localizes with endogenous frog rod PDE6 (Muradov et al., 2009). Recently, using transgenic *X. laevis* we demonstrated that the GAFa domain of PDE6 contains an OS localization signal (Cheguru et al., 2014). Here, we expressed seven known ACHM-linked missense mutants of PDE6C in rods of *X. laevis* to examine the mutations' effects on the protein expression, stability, and transport. The PDE6C mutants demonstrated distinct patterns of protein stability and compartmentalization depending on the domain(s) harboring the mutation and indicated a spectrum of mechanisms in achromatopsia.

2. Results

2.1. PDE6C with ACHM mutations in the catalytic domain is properly transported to the OS

Seven missense mutations of PDE6C linked to ACHM fall into three categories based on their sequence localization (Fig. 1). Two mutations, H602L and E790K, are situated in the C-terminal catalytic domain. Three mutations are within the regulatory N-terminal GAF domains, R104W in GAFa and Y323N and P391L in GAFb. Two mutations, R29W in the N-terminus and M455V in the region linking GAFb to the catalytic domain, are located outside the conserved domains. First, we examined expression and trafficking of the catalytic domain mutants. Human EGFP-PDE6C localizes to the OS in rods of transgenic *X. laevis* in a characteristic striated pattern (Muradov et al., 2009). Live cell imaging and imaging of retina cryosections from the H602L and E790K transgenic tadpoles revealed that both PDE6C mutants were targeted to the OS (Fig. 2). Moreover, the patterns of H602L and E790K distribution in the OS were similar to that previously observed for EGFP-PDE6C.

Due to its isoprenylation, PDE6 is a peripheral membrane protein in isotonic solution, which can be solubilized by hypotonic buffers (Baehr et al., 1979). Similarly to native PDE6, EGFP-PDE6C in transgenic frog rods is extracted from the membrane with hypotonic buffer (Fig. 3). Sequential isotonic and hypotonic extractions of transgenic eyeballs combined with immunoblotting were performed to assess the expression and the membrane binding properties of the H602L and E790K mutants. The EGFP-fused full-length H602L and E790K proteins (~130 kDa bands) were found only in the hypotonic extract fractions at comparable levels (Fig. 3). This analysis also revealed an additional EGFP-containing band of ~70 kDa present in the H602L and E790K extracts. This band was relatively minor for H602L and more prominent for E790K. Apparently, the 70 kDa band is a product of proteolysis, most likely at a single site. Thus, H602L and E790K exhibit a small and a moderate reduction in proteolytic stability, respectively.

2.2. ACM mutations in the GAF domains of PDE6C lead to a discrete proteolysis of the enzyme

The PDE6C mutants of the regulatory GAF domains, R104W, Y323N, and P391L, showed analogous localization patterns in transgenic rods. For the three mutants, EGFP-fluorescence was observed mainly in the OS (Fig. 4). However, the distribution pattern of R104W, Y323N and P391L in the OS was diffused, which is different from the striated distributions of EGFP-PDE6C, H602L and E790K. Similarly to EGFP-PDE6C, expression of the mutants in transgenic *X. laevis* was mosaic (Muradov et al., 2009), i.e. expression levels varied between transgenic tadpoles and between different rods within a single retina. However, the diffused pattern of the GAF-domain mutants was the same in weakly or brightly fluorescent rods, suggesting that the pattern was not influenced by transgene expression levels. The membrane extraction and immunoblotting revealed only an ~70 kDa band containing EGFP for R104W, Y323N, and P391L (Fig. 3). The GAF domain mutants were cleaved completely as the full-length 130 kDa proteins were not detected. Since complete cleavage was observed for the mutant with average expression comparable to that of EGFP-PDE6C (R104W) or significantly lower (P391L) (Fig. 3), the degree of proteolysis does not appear to be dependent on mutant expression levels. The regulatory N-terminal portion of PDE6C fused to EGFP appears to retain correct folding as it was trafficked to the OS.

2.3. Mutations outside conserved domains mislocalize PDE6C inside the OS

A multiple sequence alignment of rod and cone PDE6 enzymes from various species (not shown) indicates that among the two mutated residues outside the structural domains of PDE6C, M455 is more conserved. On the other hand, the R29W substitution may produce a larger impact compared to M455V as it replaces a positively charged residue with a bulky hydrophobic residue. Upon expression in transgenic *X. laevis* rods, both mutants localized primarily to the OS (Fig. 5). The distribution of R29W and M455V in the OS was diffused, similarly to the EGFP pattern of the GAF domain mutants (Figs. 4, 5). Yet, in contrast to the GAF domain mutants, M455V was resistant to proteolysis, whereas R29W showed partial proteolytic cleavage to the 70 kDa product (Fig. 3). Thus, the lack of striated pattern for R29W and M455V may reflect a specific mislocalization defect.

2.4. Localization of the catalytic fragment(s) of PDE6C

Proteolytic cleavage of mutant PDE6C, particularly the GAF domain mutants, resulted in an ~70 kDa fragment suggesting that it contains ~350–400 N-terminal aa of PDE6C fused to EGFP. Thus, the C-terminal proteolytic fragment(s) of PDE6C can be as large as 450 aa or more. To examine the proteolytic stability and trafficking of the C-terminal fragment, we introduced an epitope for monoclonal EE antibody at about 20 aa upstream of the PDE6C C-terminus (Fig. 1). The EE epitope did not interfere with the expression and correct OS targeting of EGFP-PDE6C-EE. The OS localization of EGFP-PDE6C-EE was confirmed by EGFP-fluorescence and EE-immunofluorescence (Fig. 6A). Control EE-staining of EGFP-PDE6C cryosections showed only a weak nonspecific signal in the IS (Fig. 6B). The imaging of R104W-EE mutant revealed the same pattern of diffused EGFP-fluorescence in the OS as it was observed for this mutant lacking the EE-epitope (Fig. 6A). However, no EE-immunofluorescence was detected in the OS, suggesting that the

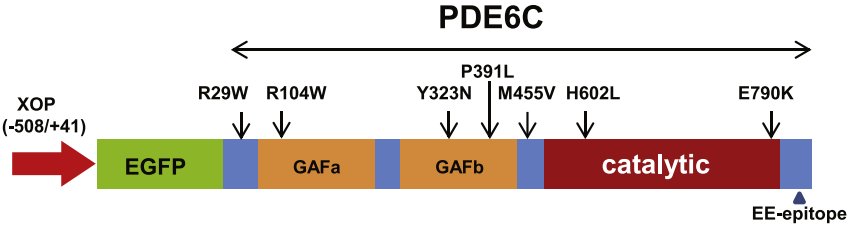

Fig. 1. Achromatopsia-linked missense mutations of PDE6C mapped on the transgenic construct for expression in rods of *X. laevis*. XOP — *Xenopus* opsin promoter.