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LIGHT/DARK TRANSLOCATION OF ALPHATRANSDUCIN IN MOUSE PHOTORECEPTOR CELLS EXPRESSING G90D MUTANT OPSIN

Zack A. Nash¹ and Muna I. Naash¹

¹Cell Biology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Blvd., Oklahoma City, OK 73104.

1. INTRODUCTION

Genomic analysis, from patients with retinal diseases such as retinitis pigmentosa (RP) and congenital night blindness (CNB), has provided convincing evidence that various subtypes of retinal diseases can result from mutations in the gene encoding rod opsin, a protein that binds 11-*cis* retinal to form the visual pigment, rhodopsin. Over 100 different mutations in this gene have been documented, and shown to co-segregate with autosomal dominant RP (Berson et al., 2002; Farrar et al., 2002; Nour and Naash, 2003) as well as a few other mutations with CNB (Dryja et al., 1993; Sieving et al., 1995; al Jandal et al., 1999). Interestingly when these CNB causing mutations are expressed in COS cells, they constitutively activated transducin in the dark while in the absence of 11-*cis* retinal (Dryja et al., 1993; Rao et al., 1994; Gross et al., 2003a; Gross et al., 2003b). Thus, the *in vitro* data suggests that the CNB mutations generate a persistent ‘dark light’ that saturates the rod photo-current and severely depresses rod sensitivity in a manner similar to that produced by light. One of these CNB mutations resulted from the replacement of glycine at position 90 by aspartic acid (G90D), which has been shown to associate with an unusual trait of congenital stationary night blindness (CSNB) (Sieving et al., 1995).

Expression of the G90D mutation in transgenic mice has recently been described (Sieving et al., 2001; Naash et al., 2004) and shown to have a persistent state of light adaptation (Sieving et al., 2001). Comprehensive studies of the functional abnormalities and the structural changes at the light- and electron-microscopic levels induced by the G90D mutation have recently been described with three transgenic lines that express the transgene at different levels (Naash et al., 2004). A direct relation between level of transgene expression and extent of photoreceptor degeneration has been observed. Higher levels of G90D opsin expression produced earlier signs of retinal degeneration and more severe disruption of photoreceptor morphology (Naash et al., 2004). Furthermore, the rod a-wave amplitudes and the amounts of photosensitive pigment were substantially reduced at early ages. Thus, these findings suggest a failure of G90D opsin to efficiently complex with the chromophore to form a light-sensitive rhodopsin in the rod outer segments (ROS). Due to the significant loss of rod sensitivity observed in these G90D retinas; this study addresses the effect of such a desensitized rod environment on the light-dependent translocation of transducin alpha subunit ($T\alpha$). Studies have shown that upon illumination, a substantial proportion of $T\alpha$ found to rapidly move out of the ROS and return only in the dark (Whelan and McGinnis, 1988). The process of $T\alpha$ movement out of the ROS seems to be mediated by the activation of transducin by photo-isomerized rhodopsin, while the reverse movement depends on the deactivation of the transduction cascade (Hardie, 2003).

2. MATERIALS AND METHODS

2.1. Generation of G90D Transgenic Mice

A 15kb mouse opsin genomic fragment was used to generate the G90D line. This fragment contains 6.0kb of the promoter region, all the introns and exons, and 3.5kb of the 3' untranslated region containing all of the multiple polyadenylation signals. Mutations were introduced by site directed mutagenesis in PCR reactions as described in previous work (Naash et al., 2004). Ten potential founders were identified and eight of them passed the transgene to their offspring. Transgenic mice were mated to rhodopsin knockout mice ($-/-$) (Lem et al., 1999) to express the transgene in different backgrounds of wild-type (wt) opsin (i.e., $+/+$, $+/-$, or $-/-$). All mice studied here were maintained in the breeding colony under cyclic light (12L:12D) conditions; cage illumination was ~7 foot-candles during the light cycle. Light-adapted animals were taken after 4 hours of being in their light cycle while dark-adapted animals were kept in the dark for at least 4-6 hours. All procedures were approved by the local Institutional Animal Care and Use Committees and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Histology and Immunohistochemistry

For histology using light microscopy, animals were sacrificed, and eyes were fixed in Davidson's fixative (95% ethanol, 10% neutral buffered formalin, glacial acetic acid, and distilled water) overnight at 4°C. The eyes were then washed 3 times in PBS and stored in 70% ethanol prior to serial dehydration and embedment in paraffin. Eyes were sectioned (6- to 8- μ m thickness) on a standard microtome and stained with hematoxylin/eosin.

For immunohistochemistry, animals were sacrificed and eyes were fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% formaldehyde. Tissues were incubated overnight with 10% sucrose in 0.1M phosphate buffer (pH 7.4) and then stored in 30% sucrose phosphate buffer. The eyes were embedded and processed as described previously (Ding et al., 2004; Nour et al., 2004). The sections were then incubated overnight at 4°C with either 1D4 (a generous gift of Dr. Robert Molday) or with a polyclonal antibody to α (purchased from Santa Cruz Biotechnology) at 1:100 dilution. Three retinal sections per slide were incubated with 1% BSA in PBS without the primary antibody as a negative control. Sections were treated with goat-anti-rabbit FITC (diluted 1:100) for 1hr at room temperature and photographed with a Zeiss universal microscope equipped for epifluorescence. Digitally captured images were saved as TIFF files on a computer and imported into Adobe PHOTOSHOP™ 8.0, where color pictures were generated.

3. RESULTS

3.1. Histological Analyses of the G90D Retinas

The G90D mutation is located in the middle of the second transmembrane domain of the molecule (Fig. 19.1). In a recently published study, a direct relation between the level of transgene expression and the extent of photoreceptor degeneration in three G90D lines was demonstrated. In this study, the histological abnormalities at one month of age in one line that expresses the transgene at a level equivalent to one wt allele was evaluated and compared to non-transgenic littermates in all rhodopsin genetic backgrounds (Fig. 19.2A (A-F)). Transgenic retinal sections on the rhodopsin wt ($+/+$) and hemi ($+/-$) backgrounds showed no obvious signs of abnormalities when compared to their cognate controls (Fig. 19.2A (A-D)). The length and organization of photoreceptor inner and outer segments as well as the thickness of the outer nuclear layer (ONL) were also similar to controls. Retinal section in Fig. 19.2A (F) shows how the incorporation of one copy of the transgene in the rhodopsin null background ($-/-$) results in the development of ROS and survival of the photoreceptors. The retinal structure of

rhodopsin null ($-/-$) animals have a normal complement of rod nuclei at early postnatal stages, but the cells fail to form ROS (Fig. 19.2B (E)).

Figure 19.2B shows the localization of the wt and mutant proteins in the G90D retinas in all rhodopsin genetic backgrounds. Using polyclonal anti-opsin antibody (1D4), which recognizes both wt and mutant opsins, on all sections from the three genetic backgrounds showed proper translocation of wt opsin as well as for the G90D protein from the site of synthesis in the rod inner segments to the disc membranes of the ROS. No labeling was seen either in the inner nuclear layer or in the cellular debris at the photoreceptor/RPE interface of rhodopsin null retina (Fig. 19.2B (F)). However, strong immunoreactivity was detected in the ROS of the G90D retinas in the rhodopsin null background (Fig. 19.2B (D)) which indicates that G90D opsin has been properly localized and integrated into the ROS disc membranes.

3.2. Light-Dependent Translocation of Transducin Alpha in the G90D Retinas

In wt mouse retinas, light triggers the translocation of transducin subunits from the outer segment in the dark, to the inner segment in the light. This process is dependent on photoisomerization of rhodopsin (Hardie, 2003). This experiment, tested whether the desensitization state of the G90D retinas interferes with the translocation of $T\alpha$ in rod cells. Comparisons of the $T\alpha$ antibody staining patterns of dark- and light-adapted wt and G90D retinas revealed that the majority of $T\alpha$ resides in the ROS of dark-adapted G90D retinas from all genetic backgrounds, whereas in the light-adapted state $T\alpha$ is found throughout the G90D photoreceptor layer, including the RIS and the synapse, similar to the distribution pattern of $T\alpha$ in non-transgenic controls. These results suggest that $T\alpha$ redistribution is not effected by the desensitization state imposed by the G90D mutation. $T\alpha$ translocation was also obvious in the G90D retinas on the rhodopsin null background (Fig. 19.3K&L). Since $T\alpha$ level in the rhodopsin null retinas is significantly low, its light-dependent translocation in the absence of rhodopsin was not apparent (Fig. 19.3I&J).

4. DISCUSSION

Light-dependent translocation of phototransduction proteins between the inner and outer segments of photoreceptor cells has been thought to play an important role in the adaptation of these cells to light (Whelan and McGinnis, 1988; Zhang et al., 2003; Elias et al., 2004; Lee and Montell, 2004). However, the mechanism by which simultaneous translocation of these proteins remains unknown. Studies have demonstrated that some of these movements occur within minutes following the onset of light (Elias et al., 2004). It has been proposed that the major function of these translocations is to provide protection of photoreceptors in bright light rather than increased vision in dim light (Elias et al., 2004).

The present study tested the light-dependent trafficking of $T\alpha$ in transgenic retinas carrying the G90D mutation in the opsin gene, a mutation that has been shown to cause a form of CSNB in humans (Sieving et al., 1995), constitutive activation of opsin in COS cells (Rao et al., 1994), and a persistent state of light adaptation ("dark light") in transgenic mice (Sieving et al., 2001; Naash et al., 2004). Since photoreceptor degeneration is directly related to the levels of transgene expression due to opsin over-expression (Naash et al., 2004), a transgenic line was used in this study that expresses G90D opsin at a level equivalent to that of wt. Although no signs of photoreceptor degeneration was seen in this line at early ages (Fig. 19.2A), retinal morphological changes were evident in older animals on different rhodopsin backgrounds (Naash et al., 2004). These changes are attributed to the presence of the G90D opsin rather than opsinoverexpression (Tan et al., 2001). As shown in Figure 19.3, the majority of $T\alpha$ resides in ROS in dark-adapted wt and G90D retinas. In the light-adapted state of the G90D retina, $T\alpha$ is found throughout the photoreceptor layer, including the RIS and the synapse, similar to the distribution of $T\alpha$ in wt mouse retinas. These results suggest that $T\alpha$ redistribution is

independent of the activation status of rhodopsin, an observation that was previously reported (Mendez et al., 2003).

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Figure 19.1.
Diagram of the mouse opsin molecule showing the location of the G90D mutation in the second transmembrane domain.

Figure 19.2.

(A). Structural analysis at the light level of one month old wt and G90D retinas on all rhodopsin genetic backgrounds. (B). Immunofluorescence of frozen sections of the same animals used in panel A labeled with 1D4. Immunofluorescence was confined almost exclusively to the ROS and rod inner segments (RIS). The fluorescent intensity appeared to be greatest in the ROS. No labeling was detected in the outer nuclear layer (ONL). Scale bar: 25 μ m.

Figure 19.3.

Sub cellular distribution of Tα in rod cells in dark- and light-adapted wt and G90D retina from all rhodopsin genetic backgrounds (+/+, +/-, and -/-). The patterns of immuno-staining in sections taken from dark- and light-adapted wt and G90D retinas are similar suggesting that the desensitization state of the G90D rods has no effect on the translocation of transducin. Scale bars: 25 μm; (insets).