



# Mutation of a Conserved Proline Disrupts the Retinal-Binding Pocket of the X-linked Cone Opsins

Harry Ostrer, Manija A. Kazmi

Human Genetics Program, Department of Pediatrics, New York University Medical Center, New York, NY

**Purpose:** To test the effects of disruption of a conserved proline in the green cone opsin molecule on light-activated isomerization, transducin activation, protein accumulation, glycosylation, and transport.

**Methods:** Stable cell lines were established by transfecting EBNA-293 cells with a plasmid containing wild-type or mutant (P307L) green opsin cDNA molecules. The proteins were induced by culturing the cells in the presence of CdCl<sub>2</sub> and analyzed by spectra, transducin activation, Western blotting, and immunocytochemistry.

**Results:** The P307L mutation diminished ability of the visual pigment to absorb light at the appropriate wavelength and to activate transducin. Protein glycosylation and transport to the cell membrane were unaffected. Although there was some diminution in the accumulation of the opsin, this was insufficient to account for the observed effect.

**Conclusions:** Like rhodopsin, the formation of the cone opsins visual pigments is dependent on the binding of retinal into a hydrophobic pocket that is formed by the second and fourth transmembranous loops. Disruption of a conserved proline near the retinal binding site represents a cause of color vision deficiency that is unrelated to spectral shifts of the photopigment.

The visual pigments are a family of proteins that mediate vision through their absorption of light (1-7). Humans have two groups of visual pigments, rhodopsin (expressed in rod cells) and the cone opsins. Rhodopsin provides monochromatic vision under low intensity light. Cone opsins provide vision under higher intensity light. There are three cone opsin pigments with short (S cone or blue), medium (M cone or green), or long (L cone or red) wavelength absorption spectra, each of which is encoded by a separate gene (8). The genes for red and green cone opsins occur as a tandem array on the distal long arm of the X chromosome. These genes are likely to have arisen from unequal recombination on the X chromosome and demonstrate 98% identity at the DNA sequence level. Recurrent recombinational events gave rise to variable numbers of red and green genes or to red/green hybrid genes (8-10). All of the visual pigments are composed of an apoprotein molecule (or opsin) that is conjugated to the chromophore, 11-cis-retinal. In response to the absorption of a photon of light, the chromophore is isomerized to all-trans-retinal. This conformational change in the visual pigment molecule causes activation of the G-binding protein, transducin.

The structure and function of the rhodopsin molecule have been extensively studied by peptide mapping and site-directed mutagenesis, and is predicted to be a protein with seven transmembrane segments. The cone opsins share 40-44% homology with rhodopsin at the amino acid level, suggesting similar structures (Figure 1) (8). Analysis molecular variants

in the X-linked cone opsin genes in individuals with heritable defects in discerning red and green hues (or color vision deficiencies) has led to the identification of the functions of specific amino acid residues. Amino acid residues that account for green/red differences in spectral tuning include A180S, F277Y, and A285T (11-17). Disulfide cross-linking between the third transmembrane helix and the second extracellular loop occurs at cysteines 126 and 203. Mutation of C203 disrupts folding and half-life of the cone opsin molecule and its abilities to absorb light at the appropriate wavelength and to activate transducin, thus causing color vision deficiencies (18). Here, we characterize mutation of the proline at residue 307 (P307), which has been observed as a cause of blue cone monochromacy, a severe form of color vision deficiency (19). Based on its conservation in rhodopsin, this residue is predicted to alter the structure of the retinal binding pocket.

## METHODS

*Test plasmids*— All site directed mutagenesis was performed on the clone, pBCGrnC1D4 (18). This clone included a full-length green cDNA, the epitope for the C-terminus of rhodopsin, and the vector pBC KS (Stratagene, LaJolla, CA). Site-directed mutagenesis was used for construction of all mutants (Transformer Site-Directed Mutagenesis Kit, Clontech Laboratories, Palo Alto, CA). The selection primer for transforming a MluI site to a HindIII site was 5'CGACGGTATCGATAAGCTTGATATCGAATTCC3'. The primer for mutagenesis (P307L) was 5'CAAAGAAGGCCAGCAGGGCAGCC3'. All mutants were verified by sequencing. For creation of stable cell lines, all constructs were subcloned into the vector, pMEP4β (provided by Dr. Mark Tyckocinski, Case Western Reserve

\*To whom correspondence should be addressed: Harry Ostrer, M.D., Human Genetics Program, Department of Pediatrics, New York University Medical Center, 550 First Avenue, New York, NY, 10016, Phone: (212) 263-5746, Fax: (212) 263-7590, email: [ostreh01@mcrcr6.med.nyu.edu](mailto:ostreh01@mcrcr6.med.nyu.edu)

University, Cleveland, OH) (20-22). The final constructs were called pGrn and pGrnP307L.

**Analytical Techniques**— Transfection, cell culture, selection, induction, Western blots, glycosidase digestion, spectra, transducin activation, and immunofluorescence were performed as previously described (18,20). All experiments were performed a minimum of three times with single replicates being shown here. Statistical analysis was performed by least squares regression and t-testing of the differences of the slopes.

## RESULTS

Mutation of a conserved proline reduces light-activated isomerization and light-dependent transducin activation. When the epitope-tagged, wild-type green opsin was expressed in 293-EBNA cells stably transfected with plasmid pGrn and reconstituted with retinal, the photobleaching difference spectrum revealed a maximal absorption of light ( $\lambda_{\max}$  value) at 530 nm, which is characteristic of green (Grn) opsins (Figure 2). By contrast, the photobleaching difference spectrum at  $\lambda_{\max}$  530 nm was reduced 8-fold for the mutant, epitope-tagged P307L green opsin prepared and bleached under identical conditions (Figure 2). The signal was consistently low in three replicates. The possibility of a small spectral shift cannot be excluded.

Light activation of bovine transducin as demonstrated by the GTP  $\gamma^{35}\text{S}$  binding assay was markedly diminished for the mutant opsin compared to the wild type, but above the baseline (Figure 3). Using least squares regression analysis, the slopes ( $\pm$  standard errors) are: Grn  $296.7 \pm 19.3$ , P307L  $50.6 \pm 5.0$ , pMEP4  $12.347 \pm 1.9$ . A t-test for the difference in slopes between P307L and pMEP4  $\beta$  was 7.13 ( $p < 0.00001$ ), and the 95% CI for the difference was 26.8 to 49.8. For the

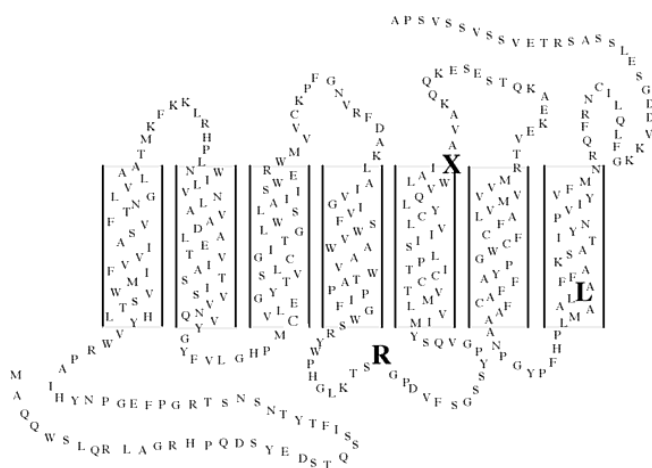


Figure 1. A secondary structure model of green cone opsin. The location of the P307L mutation is highlighted. The amino acid sequence of the 1D4 epitope is shown in bold. R indicates the substitution of arginine for cysteine at position 203. X indicates the substitution of a premature terminator for arginine at position 247.

comparison of slopes for P307L and GRN,  $t$  was 12.33 ( $p < 10^{-8}$ ) and the 95% CI was -203.2 to -288.9.

Protein glycosylation and transport to the cell membrane are unaffected by the mutation. Western blot analysis demonstrated that for a comparable number of cells that there was a slight reduction in the accumulation of the mutant cone opsin, but that this reduction was insufficient to account for the failure to generate a photoactive cone opsin (Figure 4). The presence of identical banding patterns, including the fully glycosylated form of the P307L cone opsin, suggested that there was no substantial alteration in folding or transport. To

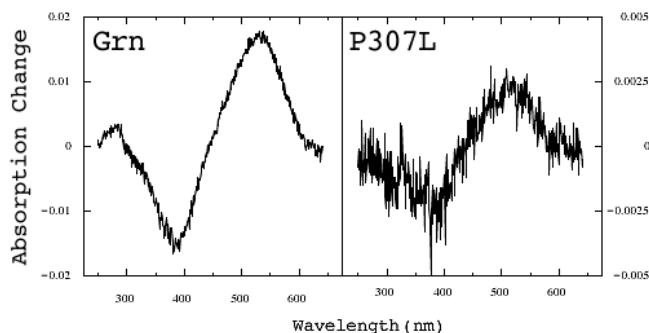


Figure 2. Photobleaching difference spectra for purified wild-type and mutant green opsins. Spectra were measured both prior to and following bleaching with light greater than 495 nm wavelength and differences were calculated. The difference spectra show that both the wild type (Grn) and mutant opsins (P307L) form visual pigments with absorption maxima at 530 nm. The scales of the type graphs are different and demonstrate markedly diminished absorption for the mutant pigment compared to the wild-type.

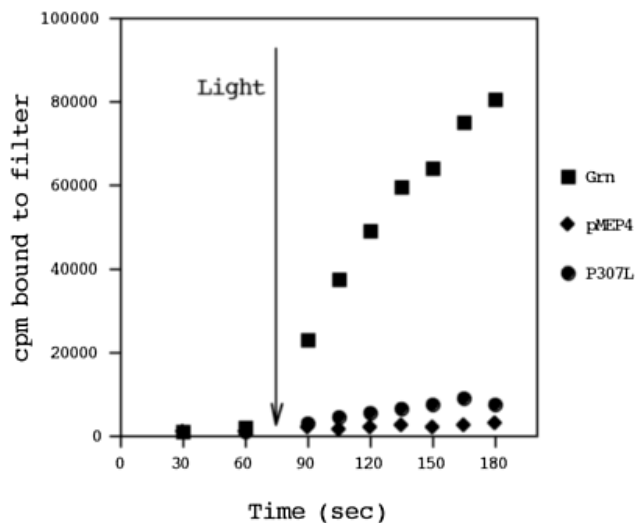


Figure 3. Light-dependent transducin activation by wild-type and mutant (P307L) green opsins. Following induction of pGrn and pGrnP307L, membrane preparations were made, then tested for their ability to bind GTP ( $^{35}\text{S}$ ) in the presence of bovine rod transducin. The mutant opsin was able to activate transducin, albeit less efficiently than the wild type.

further test whether differences occurred with glycosylation of the mutant polypeptide, cell extracts expressing the cone opsins were digested with the enzymes endoglycosidase F and endoglycosidase H. Endoglycosidase F cleaves N-linked carbohydrate residues, whereas endoglycosidase H cleaves mannose-rich, but not complex, carbohydrate moieties. No differences were observed for glycosidase digestion of the mutant and wild-type green opsin molecules (data not shown). Thus, as previously demonstrated for the wild-type green opsin, the P307L mutant opsin is N-glycosylated, initially with mannose and then with complex carbohydrate residues (18).

To further demonstrate that this mutation did not impair transport of the cone opsin molecules to the cell membrane, immunofluorescence analysis was performed. Surface, rather than cytoplasmic, staining was observed for both wild-type and mutant cone opsin molecules (Figure 5).

## DISCUSSION

Proline residues are highly conserved among the opsin genes. Sixteen of the 20 proline residues in human rhodopsin are conserved in the human red and green cone opsins. All five of the proline residues that occur in transmembranous helices are conserved between rhodopsin and the cone opsins. This includes P291 in rhodopsin and P307 in the cone opsins. This high degree of proline conservation is in keeping with its role to induce a 20 degree kink and thus to conserve the protein secondary structure (1). The presence of prolines in five of the seven transmembranous helices is thought to be important

for creating a pocket that can accommodate the chromophore, 11-cis-retinal (23). Substitution of a leucine for a proline near the presumed retinal binding site at residue 312 is likely to alter the structure of the retinal binding pocket without disrupting the helical conformation (1). As a result, reconstitution of the mutant polypeptide with retinal regenerates an inefficient visual pigment without shifting the maximal wavelength of light absorption nor altering the glycosylation or transport to the cell membrane. The effects of this proline mutation in the cone opsins are comparable to the class I proline mutations in rhodopsin, P347L and P347S, that are causes of autosomal dominant retinitis pigmentosa (24).

This observation extends the number of molecular mechanisms that have been identified as causes of blue cone monochromacy. This condition occurs when there is absence of both red and green visual pigments (7,19,25). Point mutations in a single X-linked cone opsin gene can alter its expression (Figure 1). A nonsense mutation, A247X, was found in one blue monochromat (19). The most commonly observed mutation, C203R, was found in fifteen blue cone monochromats with a single, hybrid X-linked cone opsin gene and in both X-linked cone opsin genes of one individual (19,26). This mutation alters a conserved cysteine, thereby disrupting a disulfide bond. The encoded cone opsin is folded improperly, retained in the endoplasmic reticulum and rendered unstable (18).

The other commonly observed cause of blue cone monochromacy is deletion of at least 600 bp of DNA in individuals with one or more cone opsin genes. This locus

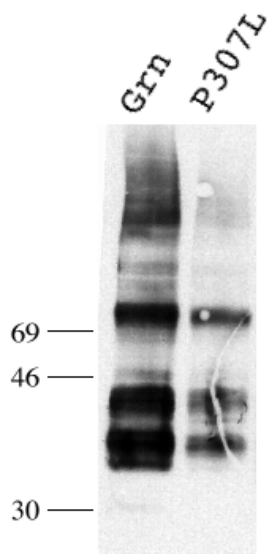


Figure 4. Western blot analysis of wild type and mutant green opsins. Polypeptides from lysates of equal numbers of cells were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with 1D4 mAb. Immunoreactive bands were visualized using chemiluminescence. The band just below the 46 kd molecular weight marker represents the fully glycosylated form. All bands above 46 kd represent multimeric forms of the protein. No differences were observed in the glycosylation patterns of the wild-type and mutant opsins.

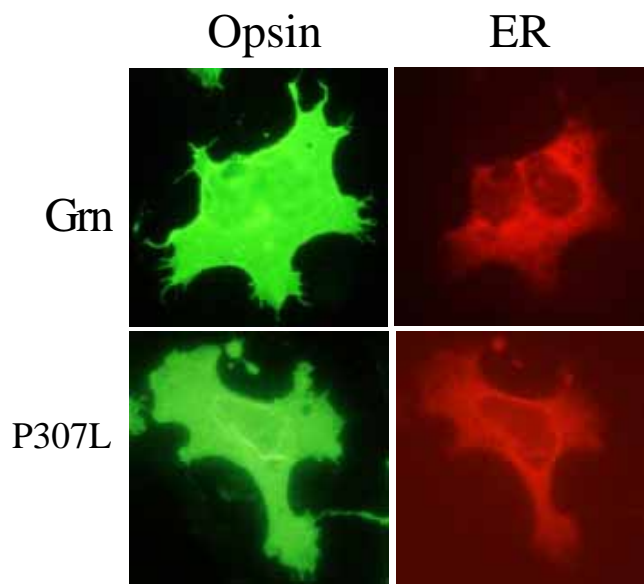


Figure 5. Immunofluorescence analysis of 293-EBNA cells expressing Grn and P307L. Cells were incubated with either the 1D4 mAb and a secondary fluoresceinated goat anti-mouse Ab (opsin) or with RIC6 and RIIL3 polyclonal antibodies and a secondary Texas red-conjugated goat anti-rabbit Ab (ER). No differences were observed in the immunofluorescence patterns of the wild type and mutant opsins, indicating that the mutant opsin is transported normally to the cell membrane.

control region (LCR) is located between 3.1kb and 3.7 kb on the 5' sides of the transcription initiation site of the first cone opsin gene (19). Deletion of the LCR silences the expression of any red, red/green hybrid, or green genes that may be present. The necessity of this region for the expression of the human X-linked cone opsin gene expression has been demonstrated in transgenic mice (27).

The cause of the blue cone monochromacy has not been determined in at least nine individuals whose X-linked cone opsin genes were studied by quantitative Southern blot analysis and DNA sequencing (19; unpublished data). Virtually all of the unknowns occurred in pedigrees in which the blue cone monochromacy was linked to the cone opsin loci. When the mutations in these individuals are discovered, they are likely to demonstrate new mechanisms for the causes of color vision deficiencies, including the identification of regions other than the LCR that affect the expression of the X-linked cone opsin genes.

### ACKNOWLEDGEMENTS

The authors thank T. Sakmar, T. Zvyaga, A. Cypess, and M. Han for advice with assays, R. Dubin and C. Oddoux for helpful discussions, and G. Kreibich for the RIC6 and the RIIL3 rabbit polyclonal antibodies and advice about immunofluorescence assays, Robert Pergolizzi for assistance with the automated DNA sequence analysis, and Roy Shore for assistance with the statistical analysis. This work was supported by NIH R01 EY08076.

### REFERENCES

- Applebury ML, Hargrave PA. Molecular biology of the visual pigments. *Vision Res* 1986; 26:1881-1895.
- Nathans J. Molecular biology of visual pigments. *Annu Rev Neurosci* 1987; 10:163-194.
- Nathans J. Protein-chromophore interactions in rhodopsin studied by site-directed mutagenesis. *Cold Spring Harb Symp Quant Biol* 1990; 55:621-633.
- Hargrave PA, McDowell JH. Rhodopsin and phototransduction: a model system for G protein-linked receptors. *FASEB J* 1992; 6:2323-2331.
- Khorana HG. Rhodopsin, photoreceptor of the rod cell. An emerging pattern for structure and function. *J Biol Chem* 1992; 267:1-4.
- Stryer L. Visual excitation and recovery. *J Biol Chem* 1991; 266:10711-10714.
- Nathans J. In the eye of the beholder: Visual pigments and inherited variation in human vision. *Cell* 1994; 78:357-360.
- Nathans J, Thomas D, Hogness DS. Molecular genetics of human color vision: the genes encoding blue, green and red pigments. *Science* 1986; 232:193-202.
- Nathans J, Piantanida TP, Eddy RL, Shows TB, Hogness DS. Molecular genetics of inherited variation in human color vision. *Science* 1986; 232:203-210.
- Vollrath D, Nathans J, Davis RW. Tandem array of human visual pigment genes at Xq28. *Science* 1988; 240:1669-1672.
- Neitz M, Neitz J, Jacobs GH. Spectral tuning of pigments underlying red-green color vision. *Science* 1991; 252:971-974.
- Neitz J, Jacobs GH. Polymorphism in normal human color vision and its mechanism. *Vision Res* 1990; 30:621-636.
- Winderickx J, Lindsey DT, Sanocki E, Teller DY, Motulsky AG, Deeb SS. Polymorphism in red photopigment underlies variation in colour matching. *Nature* 1992; 356:431-433.
- Merbs SL, Nathans J. Absorption spectra of human cone pigments. *Nature* 1992; 356:433-435.
- Merbs SL, Nathans J. Absorption spectra of the hybrid pigments responsible for anomalous color vision. *Science* 1992; 258:464-466.
- Merbs SL, Nathans J. Role of hydroxyl-bearing amino acids in differentially tuning the absorption spectra of the human red and green cone pigments. *Photochem Photobiol* 1993; 58:706-710.
- Asenjo AB, Rim J, Oprian DD. Molecular determinants of human red/green color discrimination. *Neuron* 1994; 12:1131-1138.
- Kazmi MA, Sakmar TP, Ostrer H. Mutation of a conserved cysteine in the X-linked cone opsins causes color vision deficiencies by disrupting protein folding and stability. *Invest Ophthalmol Vis Sci* 1997; 38:1074-1081.
- Nathans J, Maumenee IH, Zrenner E, Sadowski B, Sharpe LT, Lewis RA, Hansen E, Rosenberg T, Schwartz M, Heckenlively JR, et al. Genetic heterogeneity among blue-cone monochromats. *Am J Hum Genet* 1993; 53:987-1000.
- Kazmi MA, Dubin RA, Oddoux C, Ostrer H. High-level inducible expression of visual pigments in transfected cells. *Biotechniques* 1996; 21:304-311.
- Hambor JE, Hauer CA, Shu HK, Groger RK, Kaplan DR, Tyckocinski ML. Use of an Epstein-Barr virus episomal replicon for anti-sense RNA-mediated gene inhibition in a human cytotoxic T-cell clone. *Proc Natl Acad Sci U S A* 1988; 85:4010-4014.
- Hauer CA, Getty RR, Tyckocinski ML. Epstein-Barr virus episome-based promoter function in human myeloid cells. *Nucleic Acids Res* 1989; 17:1989-2003.
- Dratz EA, Hargrave PA. The structure of rhodopsin and the outer rod disk membrane. *Trends Biochem Sci* 1983; 8:128-131.
- Kaushal S, Khorana HG. Structure and function in rhodopsin. 7. Point mutations associated with autosomal dominant retinitis pigmentosa. *Biochemistry* 1994; 33:6121-6128.
- Nathans J, Davenport CM, Maumenee IH, Lewis RA, Hejtmancik JF, Litt M, Lovrien E, Weleber R, Bachynski B, Zwas F, et al. Molecular genetics of human blue cone monochromacy. *Science* 1989; 245:831-838.
- Winderickx J, Sanocki E, Lindsey DT, Teller DY, Motulsky AG, Deeb SS. Defective colour vision associated with a missense mutation in the human green visual pigment gene. *Nature Genet* 1992; 1:251-256.
- Wang Y, Macke JP, Merbs SL, Zack DJ, Klaunberg B, Bennett J, Gearhart J, Nathans J. A locus control region adjacent to the human red and green visual pigment genes. *Neuron* 1992; 9:429-440.