



Novel missense mutations in red/green opsin genes in congenital color-vision deficiencies

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Received 1 May 2002

Abstract

The DNAs from 217 Japanese males with congenital red/green color-vision deficiencies were analyzed. Twenty-three subjects had the normal genotype of a single red gene, followed by a green gene. Four of the 23 were from the 69 protan subject group and 19 of the 23 were from the 148 deutan subject group. Three of the 23 subjects had missense mutations. The mutation Asn94Lys (AAC → AAA) occurred in the single green gene of a deutan subject (A155). The Arg330Gln (CGA → CAA) mutation was detected in both green genes of another deutan subject (A164). The Gly338Glu (GGG → GAG) mutation occurred in the single red gene of a protan subject (A89). Both normal and mutant opsins were expressed in cultured COS-7 cells and visual pigments were regenerated with 11-*cis*-retinal. The normal red and green opsins showed absorbance spectra with λ_{\max} of 560 and 530 nm, respectively, but the three mutant opsins had altered spectra. The mutations in Asn94Lys and Gly338Glu resulted in no absorbance and the Arg330Gln mutation gave a low absorbance spectrum with a λ_{\max} of 530 nm. Therefore these three mutant opsins are likely to be affected in the folding process, resulting in a loss of function as a visual pigment. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Visual pigment gene; Opsin; Congenital color-vision deficiency; Protan; Deutan; Mutation; In vitro expression; 11-*cis*-Retinal; Spectrophotometry

The genes for the red/green cone opsins are present in a head-to-tail tandem manner on human chromosome X, constitute the visual pigment gene array, and are responsible for congenital red/green color-vision deficiencies. Deeb et al. [1] studied 64 color-deficient subjects and found that protan color-vision defects, in which red cones are functionally affected, were associated with a 5'-red-green hybrid gene. The deutan color-vision defects, in which green cones are functionally affected, were associated with green gene deletion or with 5'-green-red hybrid genes. Congenital red/green color-vision deficiencies have been attributed to such

gross structural rearrangements of the visual pigment gene array rather than point mutations. The single nucleotide substitution Cys203Arg (TGC → CGC) in the green genes is the only mutation so far reported [2].

We have studied the red/green opsin genes in Japanese subject groups, consisting of 121 color-normal males [3,4], 146 females [5], and 62 color-deficient males [6]. Among the color-deficient subjects, seven were thought to have the normal genotype of a single red gene, followed by a green gene(s) [6]. In our analysis of DNA from an additional 155 color-deficient Japanese males, we found that a total of 11% (23/217 subjects) had such genotypes. We sequenced all of the exons, including flanking regions, of the red/green opsin genes in these subjects and found three novel missense

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mutations. The impact of these missense mutations on opsin function was analyzed in this study.

Materials and methods

Analysis of red and green opsin genes in color-deficient males. We analyzed DNA isolated from the peripheral blood leukocytes from 217 color-deficient males, who had consulted Shiga University of Medical Science Hospital or Japan Red Cross Nagoya First Hospital. All study subjects gave informed consent. The handling procedures of these DNAs were carried out in accordance with the Declaration of Helsinki. The number and ratio of the red and green opsin genes were determined as previously described [3,4]. For subjects thought to have normal genotypes, the first gene and the downstream genes in the visual pigment gene array were amplified separately by the previously described method [5]. The exons 1–6 and their adjacent introns were amplified by the second PCR [1,6]. The products were sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In vitro mutagenesis of red and green opsin cDNAs. The red and green opsin cDNAs were obtained by PCR from the Human Retina Marathon-Ready cDNA (Clontech Laboratories, Palo Alto, CA, USA) and cloned into the *KpnI* site of an expression vector, pFLAG-CMV-5a (Sigma-Aldrich, St. Louis, MO, USA). Genes expressed by this vector are expected to have a FLAG-peptide-tag at their C-terminus. In vitro mutagenesis of the cDNAs was done by the PCR method of [7].

Transfection and regeneration of visual pigments. The opsin cDNAs (5 µg/10 cm plate, 15 plates for each cDNA) were transfected into COS-7 cells using FuGENE 6 (Roche Diagnostics GmbH, Mannheim, Germany) at a ratio of 3 µl/µg of DNA. In co-transfection experiments, 2.5 µg of each cDNA was used. Forty-eight hours after transfection, the cells were harvested in Pm buffer (50 mM HEPES-Na, pH 6.5, 0.14 M NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, and 1 µg/ml each of aprotinin and leupeptin), pelleted by centrifugation, resuspended in Pm buffer with approximately 10 nmol of 11-*cis*-retinal, and incubated for at least 5 h at 4 °C. The cells were centrifuged, homogenized in buffer E (Pm buffer containing 0.75% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and 0.8 mg/ml of phosphatidylcholine (dipalmitoyl), Sigma-Aldrich), and then centrifuged at 100,000g for 20 min at 4 °C. Absorption spectra of the supernatant before and after bleaching with light (>590 nm) were recorded using a spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan). The supernatant was also subjected to SDS-PAGE by the method of [8]. The prestained SDS-PAGE standards (broad range, Bio-Rad Lab, Hercules, CA, USA) were used as size markers. The separated proteins were blotted to a PVDF membrane (Millipore Corp, Bedford, MA, USA) using the Mini Trans-Blot Cell (Bio-Rad Lab). The opsins tagged with the FLAG-peptide at their C-terminus were detected with biotinylated anti-FLAG monoclonal antibody (M2, Sigma-Aldrich) and alkaline

phosphatase-streptavidin conjugate (Zymed Lab., San Francisco, CA, USA).

Successive extraction of expressed opsins. The transfected cells with the opsin cDNAs were pelleted by centrifugation at 800g for 5 min and homogenized in HBS/Mg buffer (50 mM HEPES-Na, pH 7.4, 0.14 M NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, and 1 µg/ml each of aprotinin and leupeptin) containing CHAPS, at 0.1%, 0.2%, 0.4%, and 0.8%, at 4 °C. Each supernatant, after centrifugation at 15,000g for 5 min, was subjected to SDS-PAGE and immunoblotting.

Comparison of vertebrate opsins. Amino acid sequences of opsins were compared among 30 vertebrates (55 opsins) as the following: human (blue (U53874) and rhodopsin), gorilla blue (AF039428), pigmy chimpanzee blue (AF039432), macaque monkey (blue (AF158977) and rhodopsin), new world monkey (red and blue), prosimian opsin, cow (red, blue, and rhodopsin), rabbit (green and rhodopsin), horse green, goat red, deer green, cat red, dog (red and rhodopsin), guinea pig green, squirrel green, hamster rhodopsin, rat (green and blue (U63972)), mouse (green, blue (U49720), and rhodopsin), blind rodent opsin (AF139726), dolphin green, seal red, bird (red, green, blue, violet, and rhodopsin), chameleon rhodopsin, gecko SWS1, alligator rhodopsin, frog (red, violet, and rhodopsin), salamander (red, blue, and violet), blind cave fish (R007, G101, and G103 [9]), fish (red, green 1, green 2, blue, violet, and rhodopsin), and lamprey rhodopsin. Their database accession numbers are shown in parenthesis for those opsins that are referred to in the Discussion section.

Results

Color-deficient subjects with normal genotypes

We have studied a total of 217 color-deficient Japanese males. Their DNA was subjected to PCR-amplification for promoter and exon 5 of the red/green opsin genes. Gene numbers in the visual pigment gene array and ratios of green genes to a red gene were estimated from analyses of promoter [4] and exon 5 [3], respectively. Thirteen subjects (3 protans and 10 deutans) were thought to have one each of red and green opsin genes and the other 10 subjects (1 protan and 9 deutans) were thought to have one red and two green opsin genes. These results were confirmed by separate analysis of the first gene and the downstream genes in the pigment gene array [5]; all 23 subjects had a red gene as the first gene and green genes as the downstream genes. We then asked whether the 4 protan and 19 deutans subjects had defects in their red genes and in their green genes, respectively. The three missense mutations that were found in these patients are shown in Table 1. The protan A89 had a mutation in the red gene,

Table 1
Profiles of three subjects with normal genotypes and missense mutations

Subject	Phenotype (matching range)	Structure of pigment gene array	Mutation (exon)
A89	Protan	Red-green	Gly338Glu (red exon 6, GGG → GAG)
A155	Deuteranopia (0–73)	Red-green	Asn94Lys (green exon 2, AAC → AAA)
A164	Deuteranopia (0–73)	Red-green-green	Arg330Gln (green exon 6, ^a CGG → CAG)

Matching range was obtained with a Nagel anomaloscope. Because the phenotype of A89 was assessed only with Standard Pseudoisochromatic Plates (Igaku-Shoin, Tokyo, Japan) and the Panel-15 test, it is not known whether the subject is protanopic or protanomalous.

^a Mutation in A164 is present in both green genes.

while the deuterans A155 and A164 both had missense mutations in the green genes. The other 20 subjects had no mutations in either the exons (including Cys203Arg [2]) or in their flanking introns, but 15 deutan subjects had a nucleotide substitution at -71 in the promoter of the green genes (A \rightarrow C).

In vitro expression of mutant opsins

Fig. 1 shows an immunoblot of the *in vitro* expressed opsins. The major band appearing in extracts of the

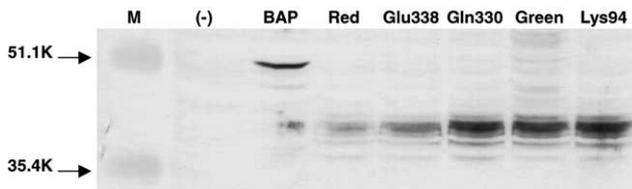


Fig. 1. *In vitro* expression of cone opsins. M, prestained protein size markers; (-), COS-7 cell extract with no transfected DNA; BAP, bacterial alkaline phosphatase (49 K) expressed as a positive control. Each cell extract with 0.75% CHAPS was loaded onto a 12% polyacrylamide gel and the separated proteins were blotted to a PVDF membrane. The expressed opsins at 39 kDa with a FLAG-tag at their C-terminus were detected with the biotinylated monoclonal antibody against the FLAG-peptide and then streptavidin-alkaline phosphatase conjugate.

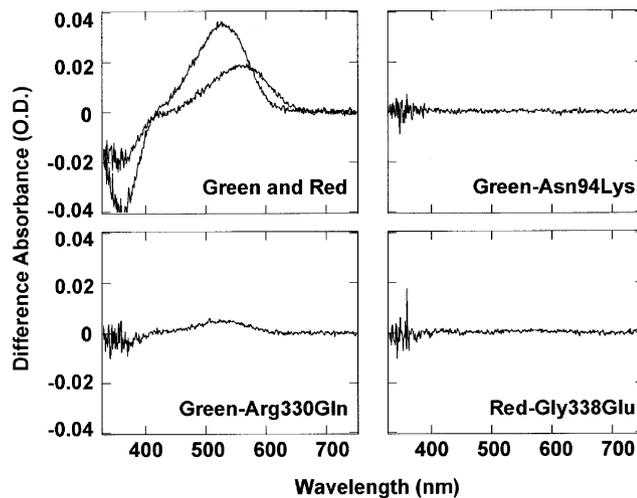


Fig. 2. Difference absorption spectra of regenerated visual pigments. The opsin cDNA, which had been cloned into an expression vector pFLAG-CMV-5a, was transfected to COS-7 cells ($5 \mu\text{g}/10 \text{ cm plate} \times 15 \text{ plates}$). Cells were harvested at 48 h and 11-*cis*-retinal was added at that time. The regenerated visual pigments were extracted with PM buffer containing 0.75% CHAPS and 0.8 mg/ml of phosphatidylcholine. Difference absorption spectra were obtained from the same samples before and after bleaching with light ($>590 \text{ nm}$). The absorption maxima (λ_{max}) were 530, 560, and 530 nm in the normal green opsin, normal red opsin, and green opsin with Gln330, respectively. No absorption was observed in the green opsin with Lys94 or in the red opsin with Glu338. In “Green and Red,” two spectra obtained for normal green and normal red opsins are superimposed.

opsins was 38 K, along with many additional bands. The expression of the mutant opsins was similar to that of the normal opsins. However, the mutant opsins in Asn94Lys and Gly338Glu showed no absorbance and mutant opsin Arg330Gln gave only low absorbance in spectrometry after regeneration of visual pigments (Fig. 2). The absorbance of the Arg330Gln mutant opsin was about 1/10 of that of the normal green opsin. To exclude the possibility that the regeneration and extraction processes of these pigments were incomplete, the mutant opsin cDNAs were co-transfected with the normal opsin cDNA. Normal green cDNA was used with the red mutant and normal red cDNA with the green mutants. In these experiments, no shift of λ_{max} from the normal opsins was observed with the mutants Asn94Lys and Gly338Glu and a slight shift of λ_{max} from the normal red (about 5–10 nm shorter) was observed for Arg330Gln (data not shown). These results indicate that the first two mutant opsins made no contribution to the spectra. Additionally, the last mutant underwent regeneration, which was roughly estimated to be 10–25% of the normal green, the 5–10 nm shift representing a mutant:normal red ratio of 1:2–5 and the expression level of normal red having been approximately half of the normal green (Fig. 2).

Extraction of expressed opsins with various concentrations of CHAPS

The following experiments were done to determine whether the expressed mutant opsins were incorporated into membrane fraction. The mutant opsins were not readily extracted with concentrations of CHAPS of 0.2% or less, but were almost fully extracted with 0.4% CHAPS (data not shown). Such CHAPS concentration-dependent extraction profiles were similar to those of normal opsin.

Discussion

Among the 217 color-deficient Japanese males, we found 23 subjects with normal genotypes. Deeb et al. [1,10] reported 1 protan and 2 deuterans with normal genotypes among a total of 92 color-deficient Caucasian subjects. Jagla et al. [11] analyzed 50 subjects who were dichromats with multiple genes in the visual pigment gene array and found five subjects with normal genotypes, three of whom had a Cys203Arg mutation. Among the additional 69 color-deficient subjects recently analyzed by our group, at least 10 subjects had normal genotypes. Therefore we are confident that color-deficient subjects with normal genotypes are much more frequent than the previous estimate of 3%. Our new estimation of a 10% or higher frequency of color-deficient individuals with normal genotypes points to an

important problem that requires solution before congenital color-vision deficiency can be accurately diagnosed.

In 3 of 23 color-deficient subjects with normal genotypes, we found missense mutations; specifically, Asn94Lys, Arg330Gln, and Gly338Glu. These mutations are all novel when compared to the blue opsin (Gly79Arg, Ser214Pro, and Pro264Ser in tritan defects [12,13]), the red/green opsin (Cys203Arg in deutan defects [2], Cys203Arg, Arg247ter, and Pro307Leu in blue cone monochromacy [14]), and rhodopsin in autosomal dominant retinitis pigmentosa and congenital stationary night blindness ([13,15–17], and OMIM180380). These mutations are summarized graphically in Fig. 3. Our study provides new functional information about the novel mutations in the cone opsins that were identified in this study.

The expressed and regenerated opsin with the Asn94Lys mutation gave no absorbance. The Asp94 residue is perfectly conserved in the 55 vertebrate opsins we compared. The crystal structure of bovine rhodopsin showed that the Asn78 (corresponding to Asn94 in the red/green opsin) is necessary for connecting helices II, III, and IV through hydrogen bonds [18]. The amino acid residues participating in the hydrogen bonds are Asn78 (II), Ser127 (III), Thr160, and Trp161 (IV). They correspond to Asn94, Ser143, Ser176, and Trp177, respectively, in the red/green opsins. It is likely that the

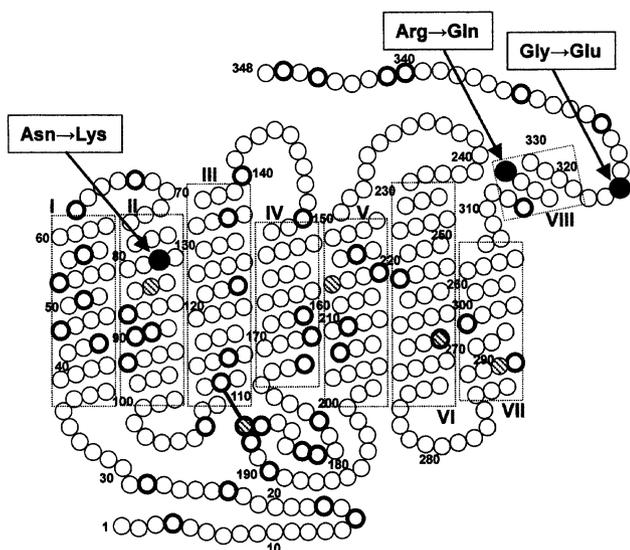


Fig. 3. Positions of missense mutations found in red/green opsins. The secondary structure of rhodopsin is shown. Arrows indicate positions of the three mutations we identified (●), at 78, 314, and 324. They correspond to 94, 330, and 338 in the red/green opsin, respectively. Positions of known mutations (excluding nonsense mutations and deletions) in rhodopsin (●) and cone opsins (⊙) are also shown. Two mutations at 187 and 267 of rhodopsin were overlapped with those of cone opsins, at 203 in green opsin and at 264 in blue opsin, respectively (⊙). The thick bar represents disulfide bonds. Boxes with dashed lines represent helices I–VIII.

same hydrogen bonds will also be important in the red/green opsins. Therefore the Asn94Lys mutation is likely to disturb the normal folding of the protein.

The opsin with the Arg330Gln mutation showed absorbance that was about 10–25% of the normal green opsin and gave no shift of λ_{\max} (530 nm, Fig. 2). It is unlikely that our results simply reflect a reduction in opsin yield, as reported by Merbs and Nathans [19], who used absorbance alone for estimating yields of hybrids between normal red and green opsins. In our study, the expression level of mutant was comparable to that of the normal green opsin by immunoblot analysis (Fig. 1). The immunoblot allows a direct visualization of expressed proteins as well as an estimate of the quantity. Therefore the low absorbance we observed is consistent with the interpretation that the mutant opsin regenerated inefficiently after addition of the 11-*cis*-retinal. In our preliminary experiments, mutant rhodopsin with Arg313Gln, corresponding to 330 in the red/green opsin, gave an absorbance spectrum similar to normal rhodopsin, suggesting a different stabilizing mechanism between cone opsins and rhodopsin.

The amino acid residue at 330, which corresponds to 321 in the blue opsin and 314 in rhodopsin, was mostly Arg in the 55 vertebrate opsins we compared. The exceptions were the blue opsins of humans, apes, and old world monkeys, where the residue is Gln, the same amino acid residue as in the mutant. We cannot explain how the blue opsins of humans, apes, and old world monkeys retain their functional state, while bearing a Gln residue at the position. The green gene of a blind rodent also has a Gln residue at this position, but we are uncertain whether the gene is functional.

In the crystal structure of bovine rhodopsin [18], helix VIII, encompassing Gln312 through Leu321 corresponding to Gln328 through Leu336 in the red/green opsin (Fig. 3), lies perpendicular to helix VII. The helix VIII region is thought to interact with G proteins. Therefore, the Arg330Gln mutation may not only disturb normal folding of the protein, but may also affect interaction of helix VIII and transducin in red/green cones.

The expressed and regenerated opsin with the Gly338Glu mutation gave no absorbance. The Gly338 residue is well conserved in the 55 vertebrate opsins we compared. The exceptions were the blue opsins of rat and mouse, in which the residue at 321, corresponding to 338 in the red/green opsin, is Arg. In our preliminary experiments, mutant rhodopsin with Gly324Glu, corresponding to 338 in the red/green opsin, gave an absorbance spectrum similar to normal rhodopsin, but green opsins with a mutation of Ala338 or Gln338 gave no absorbance. Since Gly338 may link helix VIII and the cytoplasmic domain (Fig. 3), it has not been considered to be important. However, our results indicate that this residue is essential, at least for normal folding of cone opsins, although the mechanism is unclear.

In autosomal dominant retinitis pigmentosa and congenital stationary night blindness, many mutations in the rhodopsin gene have been identified ([13,15–17], and OMIM180380) and most of them have also been functionally characterized. Among a total of 34 rhodopsin mutants [15,16], five mutants designated as class I, gave absorption spectra similar to normal rhodopsin. An additional mutation (Gly51Val), also assigned to class I gave only a 30% absorbance when compared to normal expected values, even though it was well expressed in transfected cells. The other 28 mutants, designated as class II, gave almost no absorbance. The Asn94Lys and Gly338Glu mutations resemble class II mutations of rhodopsin. The Arg330Gln mutation has a profile similar to that of the Gly51Val mutation in rhodopsin.

In conclusion, we found that more than 10% of Japanese color-deficient subjects had normal genotypes. These data suggest that some of the congenital color-vision deficiencies are caused by point mutations in the red/green opsin genes. Three amino acid residues were identified that were important for the opsins to function as the cone visual pigments. Our study provides useful information for understanding the structure–function relationships of cone opsins and rhodopsins.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14571667 to Y.S. and 14770953 to S.O.). We express our sincere gratitude to Professor Iwao Ohkubo at Shiga University of Medical Science (Department of Medical Biochemistry), Professor Kazutaka Kani at Shiga University of Medical Science (Department of Ophthalmology), and Emeritus Professor Akio Iwashima at Kyoto Prefectural University of Medicine (Department of Biochemistry) for their helpful advice and discussions. We are grateful to Mr. Masashi Suzuki at Central Research Laboratory, Shiga University of Medical Science, for his technical assistance (sequencing of DNA).

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