Mutation of a Conserved Cysteine in the X-Linked Cone Opsins Causes Color Vision Deficiencies by Disrupting Protein Folding and Stability

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Purpose. To test the effects of disruption of a conserved cysteine in the green cone opsin molecule on light-activated isomerization, transducin activation, folding, transport, and protein half-life.

Methods. Stable cell lines were established by transfecting 293-EBNA cells with a plasmid containing wild-type or mutant (C203R, C203S, C126S, C126S/C203S) green opsin cDNA molecules. The proteins were induced by culturing the cells in the presence of cadmium chloride and analyzed by spectra, transducin activation, Western blotting, pulse-labeling with immunoprecipitation, and immunocytochemistry.

Results. The C203R mutation disrupts the folding and half-life of the green opsin molecule and its abilities to absorb light at the appropriate wavelength and to activate transducin. Similar disruption of folding, half-life, and light activation occurs when Cys203 or its presumed partner for formation of a disulfide bond (Cys126) is replaced by serine residues.

Conclusions. Like rhodopsin, the folding of the cone opsins appears to be dependent on the formation of a disulfide bond between the third transmembrane helix and the second extracellular loop. Disruption of this disulfide bond represents a cause of color vision deficiencies that is unrelated to spectral shifts of the photopigment. Invest Ophthalmol Vis Sci. 1997;38:1074–1081.

The visual pigments are a family of proteins that mediate vision through their absorption of light.[1–7] 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. The resulting conformational change in the visual pigment molecule results in the activation of a G protein, transducin.

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 opsin is encoded by a separate gene.[8] The genes for red and green cone opsins are found in a tandem array on the distal long arm of the X chromosome.[8–10]

The structure and function of the rhodopsin molecule have been studied extensively by peptide mapping and site-directed mutagenesis. Comparable structural analyses have not been performed on cone opsins. At the amino acid sequence level, the degree of homology between rhodopsin and the cone opsins is 40% to 44%.[8] Based on sequence analysis, a protein with seven transmembrane segments is predicted (Fig. 1). Regions of structural similarity between the X-linked cone opsins and rhodopsin include a lysine at position 312, which is predicted to form a Schiff base with retinal, and cysteines at residues 126 and 203 that could function as sites of disulfide cross-linking.[7,11–13]

Mutational analysis of the rhodopsin gene has proved to be a powerful tool for studying the structure

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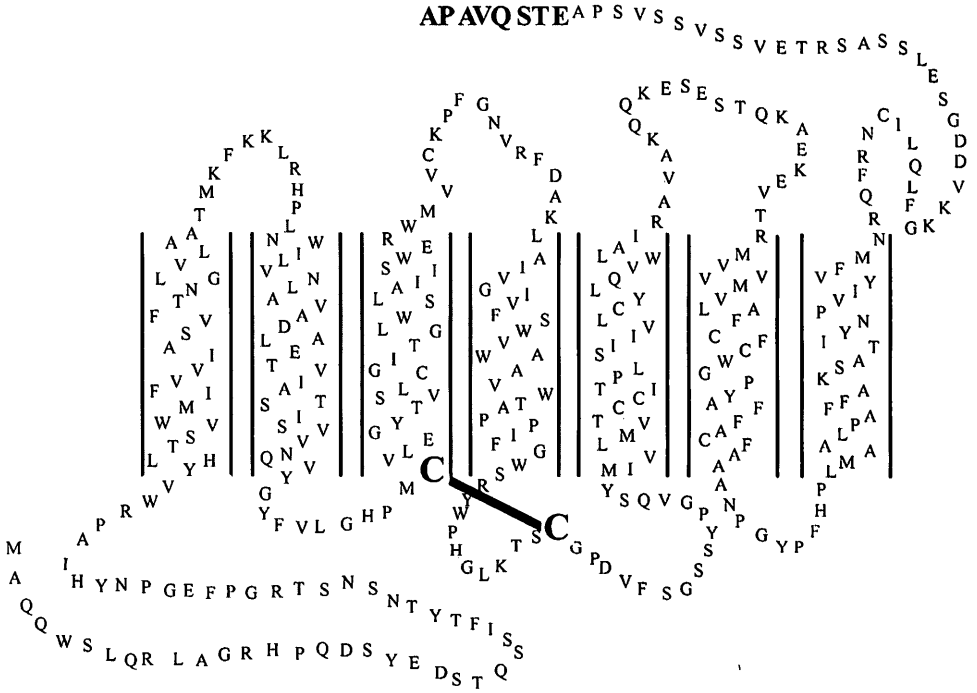

FIGURE 1. A secondary structure model of green cone opsin. The line between Cys126 and Cys203 indicates proposed disulfide bridge. The 1D4 epitope sequence is the last eight amino acids in bold.

and functions of the encoded polypeptide. Some of these mutations have been identified in persons with congenital stationary night blindness or the progressive condition, retinitis pigmentosa. Analysis of the red and green cone opsin genes has been more limited. These genes are 98% homologous to each other at the DNA sequence level and are likely to have arisen from unequal recombination on the X chromosome.[8,9] Subsequent recombinational events gave rise to variable numbers of red and green genes or to red–green hybrid genes. Analysis of these hybrid genes led to the identification of the amino acid residues that account for the spectral tuning of these proteins.[14–20]

Persons with color vision deficiencies, or color-blindness, have difficulty discerning hues in certain regions of the spectrum. These can be identified by tests of color-matching, and they fall into severe (dichromat), less severe (extreme anomalous trichromat), and mild (simple anomalous trichromat) groups.[21–23] Overlap can occur between dichromats and extreme anomalous trichromats. Dichromacy occurs when there is absence of a red or green visual pigment. Anomalous trichromacy is postulated to result from substitution of one of the red or green pigments with an anomalous pigment that has altered spectral absorption.

This explanation applies to most, but not all, persons with color vision deficiencies. Mutation of a conserved cysteine (C203R) was found in the green pigment genes of two persons with anomalous trichromacy, leading to the hypothesis that their color vision deficiencies resulted from decreased accumulation of the green cone opsin photopigment.[13] Reduced accumulation of the photopigment also has been evoked to explain the absence of red and green color vision ("blue cone monochromacy") of 15 persons with the C203R mutation in a single, hybrid X-linked cone opsin gene and 1 person with this condition whose 2 X-linked cone opsin genes have this mutation.[24,25] Here we show that the C203R mutation alters the folding and half-life of the cone opsin molecule, thereby disrupting its ability to bind retinal, to form a pigment, and to activate transducin.

METHODS

Test Plasmids

To clone a wild-type green opsin cDNA, poly (A+) RNA was extracted from cadaveric human retinas (obtained from the New York Eye Bank) by acid guanidinium extraction (Stratagene, La Jolla, CA). The green opsin cDNA was amplified by reverse transcriptase–polymerase chain reaction (Perkin–Elmer, Norwalk, CT) using primers that are specific to the 5′ and 3′ ends of the mRNA:

5′ primer GGGGATCCTCCATAGCCATGGCCCAGCA

3′ primer GCTCTAGAGGGAAAGGAGAGGTGGCCAA

The 1.29-kb polymerase chain reaction product was cloned into the pCRII vector (Invitrogen, San Diego, CA). Single colonies were sequenced using dideoxy chain termination in their entirety to confirm

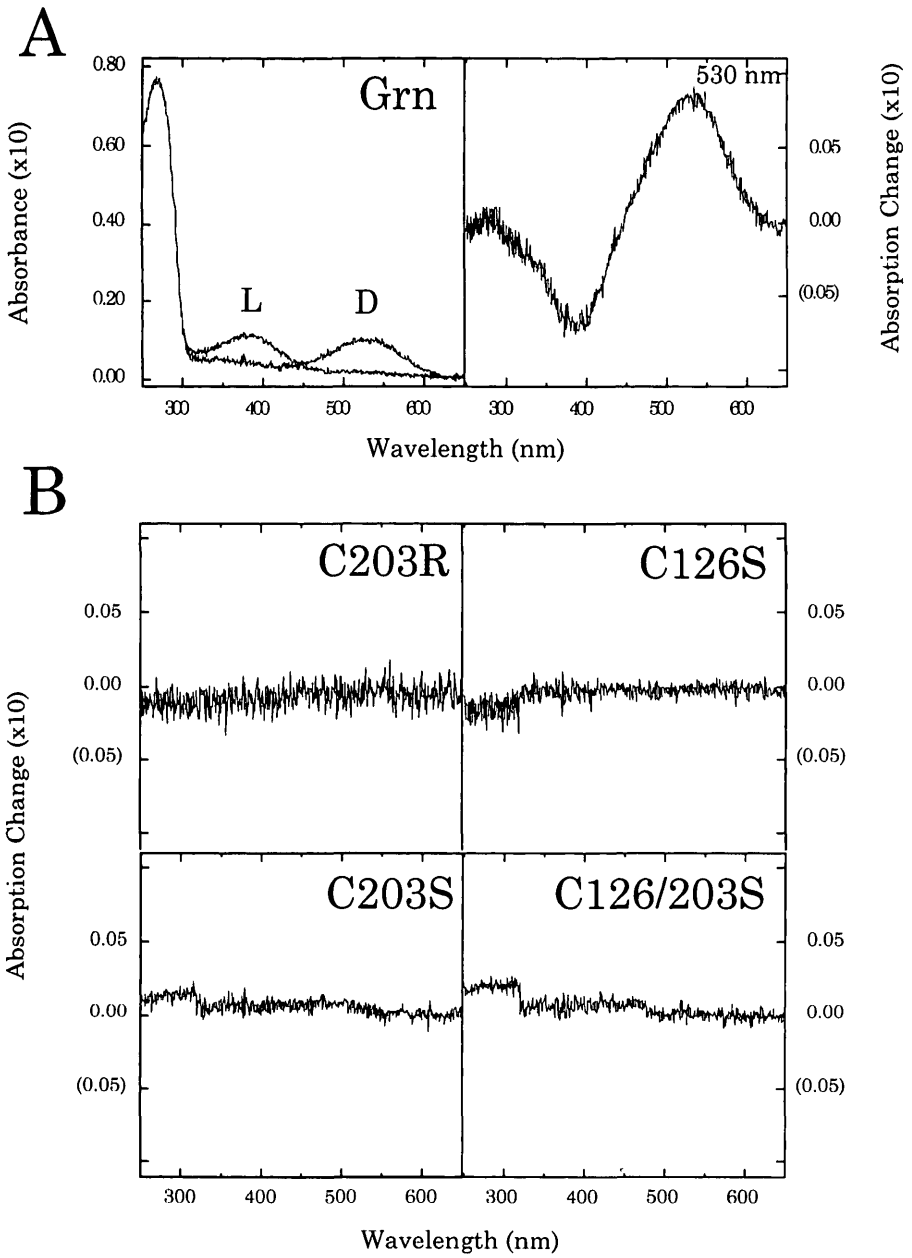

FIGURE 2. Ultraviolet–vis spectroscopy of wild-type and mutants green opsins. (**A**) Absorption spectra of Grn. Left panel shows the dark (D) and light (L) spectra superimposed. Right panel shows the difference spectra with the λ_{max} at 530 nm. (**B**) Difference spectra for green mutants C203R, C126S, C203S, and C126/203S.

the identity of the clones (U.S. Biochemical, Cleveland, OH).

For mutagenesis, the green cDNA was cloned into the EcoRI site of pBC KS (Stratagene, LaJolla, CA). To clone the 1D4 epitope tag, the 3′ HincII fragment of pBC–Grn was replaced with the 3′ HincII fragment of pBS-hs7C1D4.[26] The resulting clone was called pBCGrnC1D4. 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 primers for mutagenesis were:

C203R, 5′GAACACGTCTGGGCCGCGTGAAGTCTTCAGGCC3′

C203S, 5′GAACACGTCTGGGCCTGATGAAGTCTTCAGGCC3′

C126S, 5′GTAGCCCTCCAGGACTGACATAGGGTGGCCCAG3′

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 University, Cleveland, OH).[26–28] The final constructs were called pGrn, pGrnC203R, pGrnC126S, pGrnC203S, and pGrnC126S/C203S.

Transfection, cell culture, selection, induction, Western blots, pulse-labeling, immunoprecipitation, spectra, transducin activation, and immunofluorescence were all performed as described previously.[26]

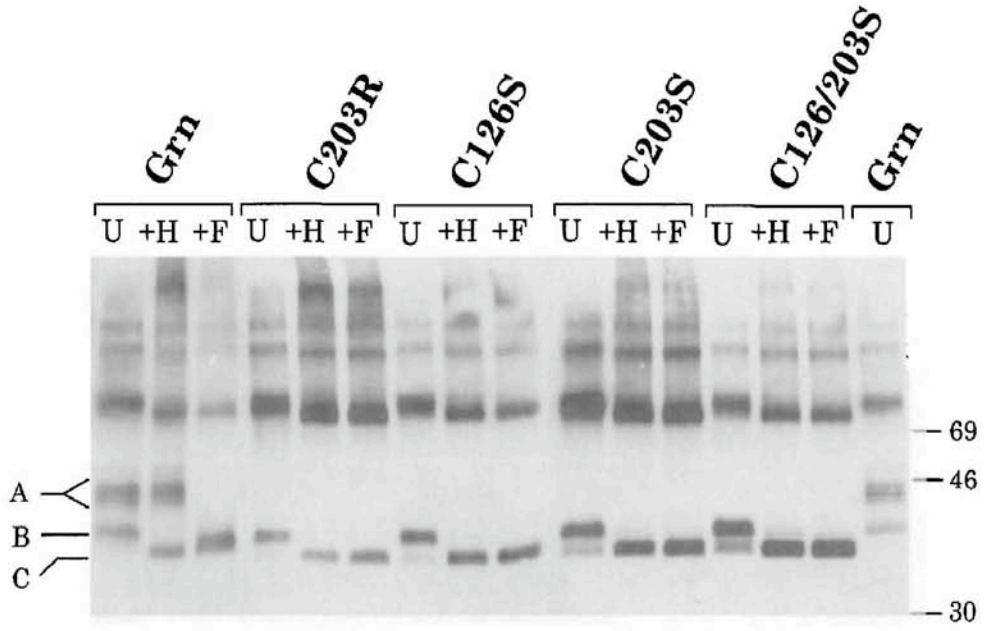

FIGURE 3. Sensitivity of opsins to endo H and endo F. Samples Grn, C203R, C126S, C203S, and C126/203S were divided into three 5-μg aliquots: one fraction was untreated (U), one fraction was treated with endoglycosidase H (+H) to remove high-mannose carbohydrates, and one fraction was treated with endoglycosidase F/N-glycosidase F (+F) to remove all N-linked carbohydrates. After treatment, samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose, and probed with 1D4 monoclonal antibody. Immunoreactive bands were visualized using chemiluminescence (ECL). The differentially glycosylated bands are indicated as (A) complex carbohydrates, (B) high-mannose, and (C) deglycosylated form. All bands above 46 kDa represent multimeric forms of the protein.

To determine the half-lives of polypeptides, the amount of radioactive counts in the ^{35}S-labeled immunoprecipitates was plotted using least mean squares log–linear regression. The calculated half-maximal value was taken to be equivalent to the half-life. To test the effects of 11-*cis*-retinal on the half-life of the wild-type green opsin, labeling was performed in 0.2 mCi ^{35}S L-methionine, 40 mM 11-*cis*-retinal in methionine-free Dulbecco's minimum essential medium with 2 mM L-glutamine for 30 minutes in the dark. All subsequent steps up to lysis were performed in the dark. The samples were chased for 0, 3, 6, 24, and 48 hours and processed as described previously.[26]

Glycosylation

Lysates were prepared as described previously.[26] Five milligrams of lysate was mixed with an equal volume of digestion buffer (1 mM calcium chloride, 20 mM ethylenediaminetetraacetic acid, 1% dodecyl maltoside, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 20 mg aprotinin, and 1.4 mg/ml pepstatin A. Samples then were treated with either 0.05 U endoglycosidase F/N-glycosidase F or 4 mU endoglycosidase H (Boehringer–Mannheim, Indianapolis, IN) for 3 hours at 37°C, then analyzed by Western blot.

RESULTS

Mutation of a Conserved Cysteine Eliminates Light-Activated Isomerization and Light-Dependent Transducin Activation

The epitope-tagged, wild-type green opsin was expressed in 293-EBNA cells stably transfected with plasmid pGrn, then reconstituted with retinal. The photobleaching difference spectrum showed a maximal absorption of light (λ_{max} value) at 530 nm, which is characteristic of green opsins (Fig. 2A). By contrast, no absorption change was observed at any wavelength for the mutant, epitope-tagged C203R green opsin prepared and bleached under identical conditions (Fig. 2B). As shown by the GTPγ(^{35}S) binding assay, the wild-type green opsin activated bovine transducin in response to light, whereas the mutant opsin did not (data not shown).

Mutation Appears to Disrupt a Disulfide Bond

Previously, it was shown that the cysteines at residues 110 and 187 of the rhodopsin molecule form a disulfide bond. Based on the sequence similarity, it was hypothesized that the cysteines at residues 126 and 203 of the red and green cone opsins also form a disulfide bond.[13,25] To test this hypothesis, mutant

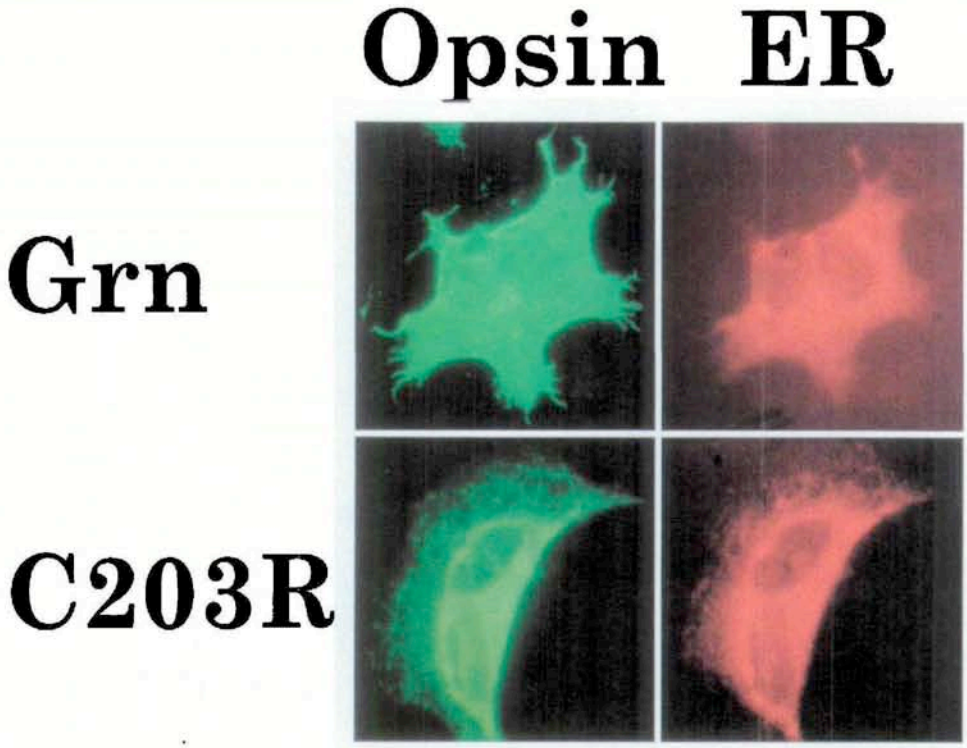

FIGURE 4. Immunofluorescence analysis of 293-EBNA cells expressing Grn and C203R. Cells were incubated with either the 1D4 monoclonal antibody and a secondary fluoresceinated goat antimouse antibody (opsin) or with RIC6 and RIIL3 polyclonal antibodies and a secondary Texas red-conjugated goat antirabbit antibody (endoplasmic reticulum).

green opsin molecules that contained disulfide bond-disrupting serines at either one or both of those positions were expressed in stable cell lines. For each of the mutants, pigment formation was not observed as judged by photobleaching difference spectroscopy (Fig. 2B). These findings are consistent with the prediction that a disulfide bond between the cysteines at residues 126 and 203 stabilizes the opsin structure capable of binding retinal.

Mutant Opsin Molecules Are Improperly Folded and Retained in the Endoplasmic Reticulum and Not Targeted to the Cell Membrane

Western blot analysis showed that the failure to generate a photoactive cone opsin was the result of impaired folding rather than impaired expression (Fig. 3). Previously, it was shown that improperly folded opsin molecules accumulate mannose-rich carbohydrates rather than the complex form of carbohydrates.[29] To test whether the mutant cone opsin post-translational product had accumulated mannose-rich carbohydrates, cell extracts expressing the cone opsins were digested with the enzyme endoglycosidase H. This enzyme is unable to cleave complex carbohydrate moieties, but it efficiently cleaves the mannose-rich form (Fig. 3). For both the wild-type and the mutant green opsin molecules, the partially processed polypeptide (corresponding to band B in Fig. 3) was digested. A fully glycosylated polypeptide that was present only for the wild-type (corresponding to band A in Fig. 3) was resistant to digestion. Digestion by the enzyme, endoglycosidase F, showed that both the wild-type and the mutant cone opsins were N-glycosylated and thus sensitive to the enzyme.

To show that the cysteine mutations impaired transport of the cone opsin molecules, immunofluorescence analysis was performed. Expression was observed for both wild-type and mutant cone opsin molecules. Surface staining was observed for the wild-type green opsin. By contrast, the staining was cytoplasmic for the C203R green opsin. Comparison of this signal with that generated by the RIC6 and RIIL3 antibodies, which are specific to ribophorin proteins in the endoplasmic reticulum, showed that the mutant opsin was retained in the endoplasmic reticulum (Fig. 4).

Stability of Mutant Opsin Molecules Is Diminished

To test whether conjugation with the chromophore during opsin synthesis alters the stability of the cone opsin molecules, pulse-chase analysis with immunoprecipitation and counting or electrophoresis and autoradiography was performed in the presence and ab-

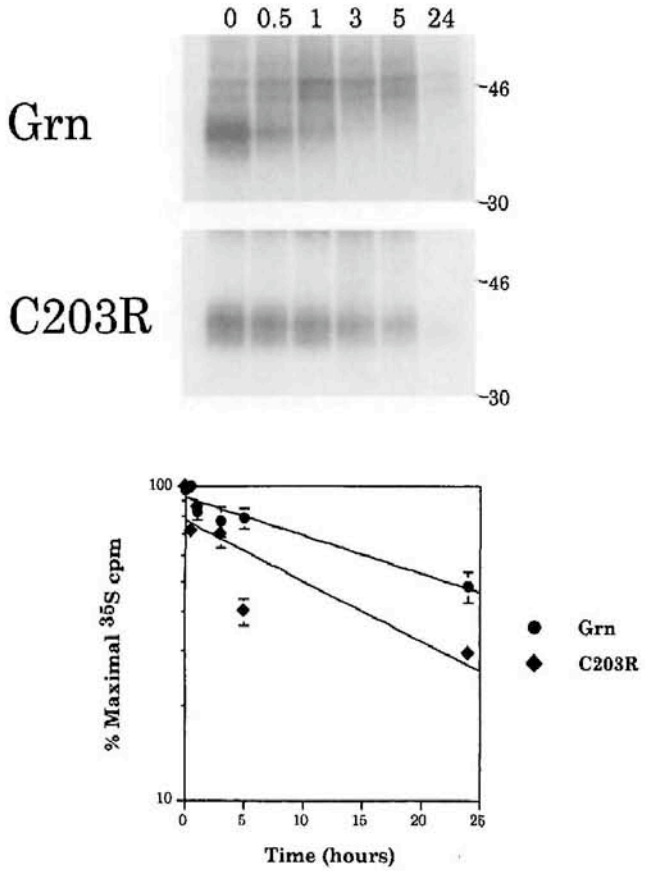

FIGURE 5. Pulse-chase analysis of Grn and C203R. Cells were treated as described in Materials and Methods. Equal volumes of immunoprecipitated material were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by autoradiography as shown in the top panel. Bottom panel shows percent maximal immunoprecipitated materials at each of the timepoints with means and standard errors of the mean for Grn (*circles*) and C203R (*diamonds*) indicated. The mutant C203R remains as a 39-kDa band, whereas the wild-type green becomes fully glycosylated with a complex pattern within 1 hour.

sence of 11-*cis*-retinal (data not shown). As calculated from the immunoprecipitation results, the half-life of the wild-type opsin molecule in the presence of retinal was 23 ± 1.6 hours and in the absence of retinal was 24.7 ± 1.7 hours ($P = 0.254$). Based on this observation, pulse-chase analysis was performed in the absence of 11-*cis*-retinal to determine whether the stability of the wild-type green opsin was comparable to rhodopsin (data not shown). The half-life of the wild-type cone opsin molecule was 28.7 ± 3 hours, whereas that of rhodopsin was 26.7 ± 3.3 ($P = 0.64$). In addition, pulse-chase analysis was performed in the absence of 11-*cis*-retinal to determine whether the stability of the mutant C203R green opsin was diminished compared with that of wild-type (Fig. 5). The half-life of the wild-type cone opsin molecule was 28.7 ± 3 hours, whereas that of the mutant was 15.8 ± 1.6 hours ($P = 0.015$).

DISCUSSION

These studies suggest that the cone opsins have a disulfide bond between the third transmembrane helix and the second extracellular loop. Disruption of the disulfide bond in either class of opsins alters folding and transport of the protein and shortens its half-life. Disruption of transport from the endoplasmic reticulum to the Golgi has been found to be the common effect of 85% of mutations in the rhodopsin gene that produce retinitis pigmentosa and have been referred to as class II mutations.[30,31] Mutations that disrupt folding are a common cause of genetic disease, having been observed for cystic fibrosis, von Willebrand disease, alpha-1-antitrypsin deficiency, factor XIII deficiency, nephrogenic diabetes insipidus, thyroxine-binding globulin deficiency, Glanzmann thrombasthenia, type I hereditary angioneurotic edema, and Tay–Sachs disease.[32–40]

The in vitro data suggest that in persons who do not have compensatory normal genes, the presence of the C203R mutation is sufficient to produce color vision deficiencies. This is a novel mechanism unrelated to alteration of spectral tuning of the encoded pigments. These findings readily explain the absence of red and green color vision for the persons with blue cone monochromacy, because these persons have a single X-linked cone opsin gene and mutation of the conserved cysteine results in expression of an opsin that is folded improperly.[24,25] This contrasts with another mechanism that has been associated with blue cone monochromacy, deletion of a locus control region that is thought to diminish the expression of the X-linked cone opsin genes.[41]

These findings also may explain the presence of deuteranomaly in persons who have this mutation in at least one of their green opsin genes. Three such persons have been described.[13] The first had extreme deuteranomaly and three green genes, all of which contained the mutation. Based on this observation, his cone cells would accumulate little, if any, photopigment. The second person had three green opsin genes and mild deuteranomaly, whereas the third had five green opsin genes and normal color vision. The C203R mutation was present in only one of their green genes. These findings have been interpreted to mean that reduced accumulation of the green opsin pigment is associated with deuteranomaly. There are two ways that this could occur. First, gene dosage, as well as position in the gene array, may affect the level of photopigment expression. Second, when expressed, the mutant gene product may have a dominant negative effect, diminishing the accumulation of expression of the wild-type photopigment.

As suggested previously, cone opsin mutations, including the C203R mutation, may play a role in retinal

degeneration.[24,42,43] The C203R mutation is similar to the class II mutations in the rhodopsin gene (including C187Y) that have been associated with retinitis pigmentosa, a progressive disorder associated with cell death.[29–31,43,44] These mutations are nonconservative and disrupt protein folding and transport. One class II mutation, P23H, has been shown to cause retinitis pigmentosa in transgenic mice.[45] If the C203R mutation caused premature cell death, this would explain the progressive central retinal degeneration that occurs in some people with blue cone monochromacy.[24] The expression of a mutant C203R green opsin may likewise predispose to cone dystrophies. This hypothesis can be tested in transgenic mouse lines that express the mutant human cone opsin.

Key Words

color vision, cone opsin, G protein, photoreceptor genetic diseases

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