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Glycosylation and Palmitoylation Are Not Required for the Formation of the X-Linked Cone Opsin Visual Pigments

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Purpose: This study was designed to test whether palmitoylation and glycosylation are required for the formation of the green opsin visual pigment.
Methods: Stable cell lines were established by transfecting EBNA-293 cells with a pMEP4β recombinant plasmid containing wild-type bovine rhodopsin or wild-type or mutant (N32S) green opsin cDNA molecules that included a tag for the eight amino acid residues located at the C-terminus of rhodopsin. The opsins were induced by addition of $CdCl_2$ into the medium and then reconstituted with 11-cis-retinal. The reconstituted opsins were purified by immunoaffinity chromatography, then analyzed by difference spectra, and by binding ^{35}S-GTP in the presence of bovine transducin. Non-reconstituted opsins were analyzed by Western blotting and by pulse-labeling with ^{3}H-palmitic acid followed by immunoprecipitation.
Results: Elimination of glycosylation by mutagenesis of the N-linked glycosylation site did not impair the ability of the resulting cone opsin to absorb light at the appropriate wavelength nor to activate transducin. Furthermore, as judged by pulse-labeling with ^{3}H-palmitic acid and immunoprecipitation and by gas chromatography-mass spectroscopy, the wild type green opsin differs from rhodopsin by not being palmitoylated.
Conclusions: Glycosylation and palmitoylation are not required for the formation of cone opsin visual pigments. For the previously described green opsin C203R mutation, disruption of folding and transport, rather than altered glycosylation is sufficient to explain the associated color vision deficiency.

The visual pigments are a family of photoreceptor proteins that absorb light and mediate vision [1-7]. Humans have two groups of visual pigments, rhodopsin, which is expressed in rod cells and provides monochromatic vision under low intensity light, and the cone opsins, which provide color 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 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, which may contain varying copy numbers of red, green and red/green hybrid genes; the blue opsin gene is a single-copy locus located at 7q22-qter [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 (The structure for the green opsin gene is shown in Figure 1) [8]. 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 function as sites of disulfide cross-linking [11-15]. The comparable sites in rhodopsin are a lysine at position 296 and cysteines at residues 110 and 187 [12].

Previously it has been shown that both rhodopsin and the X-linked cone opsins are N-linked glycosylated and that rhodopsin is palmitoylated [16-20]. Rhodopsin is glycosylated at residues N2 and N15, although glycosylation only at asparagine-15 is required to form a visual pigment. Here, we show that elimination of the N-linked glycosylation does not impair the ability of green opsin to form a visual pigment and that the wild-type green opsin is not palmitoylated.

METHODS

Plasmids: Recombinant plasmids included a full-length, synthetic bovine rhodopsin cDNA (pRho), a mutant bovine rhodopsin cDNA (pRhoC187R), and a full-length green cDNA (pGrn) that included the epitope for the C-terminus of rhodopsin [15,16,21]. Alteration of the N-linked glycosylation site from asparagine to serine at residue 32 (N32S, see Figure 1) was performed by site-directed mutagenesis (Transformer Site-Directed Mutagenesis Kit, Clontech Laboratories, Palo Alto, CA). The selection primer for transforming a MluI site to a HindIII site was 5'-CGACGGTATCGATAAGCTTGATATC GAATTCC-3'. The primer for mutagenesis (N32S) was 5'-CTCTGGTGGACTGGCTGTTGGTG-3'. All mutants were verified by sequencing. Recombinant cDNAs were subcloned into the vector, pMEP4β (provided by Dr. Mark Tyckocinski, Case Western Reserve University, Cleveland, OH), for cre-

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ation of stable cell lines [16,22,23]. The final constructs was called pGrn-N32S.

Transfection, cell culture, selection, induction, western blots, glycosidase digestion, spectra, transducin activation, and immunoprecipitation were performed as described previously [15,16]. To inhibit glycosylation, EBNA-293 cells stably transfected with pGrn or pGrn-N32S were treated with 0.5 µg/ml tunicamycin (Sigma-Aldrich, St. Louis, MO) during the 16-hour induction with $CdCl_2$. To test for palmitoylation, EBNA-293 cells stably transfected with the rhodopsin or cone opsin plasmids were induced with 5 µM $CdCl_2$, then labeled with in the presence of 0.1 mCi ^{3}H-palmitic acid (New England Nuclear, Beverly, MA). Induction and labeling were for 16 hours.

Fatty acid extraction and analysis were based on a previously described method [19]. The fatty acid methyl esters were extracted with hexane and an aliquot was analyzed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (0.25 mm x 30 m, coated with BD225 at a film thickness of 0.25 µm, J & W Scientific, Folsom, CA). The initial column temperature was 182 °C and programmed at 2 °C/min to a final temperature at 220 °C. Under the condition, methyl palmitate eluted at 8.95 min and methyl eicosanoic (an internal standard) eluted at 22.06 min. The quantity of palmitic acid was normalized to the initial amount of protein from which it was extracted [19].

RESULTS

Glycosylation is not required for formation of a green opsin visual pigment: The epitope-tagged, wild-type and N32S green opsins were expressed in 293-EBNA cells. As judged by Western blot analysis, the N32S mutant was not glycosylated (Figure 2A). Treatment with tunicamycin, which inhibits N-linked glycosylation, collapsed the pattern of the wild type green opsin and did not affect the pattern of the N32S mutant (Figure 2B). The photobleaching difference spectrum revealed a maximal absorption of light (λ_{max} value) at 530 nm for both the wild type and N32S opsins, demonstrating that the unglycosylated cone opsin forms a visual pigment that is comparable to the wild type (Figure 3). The increased noise observed in the N32S mutant tracing suggests the possibility that

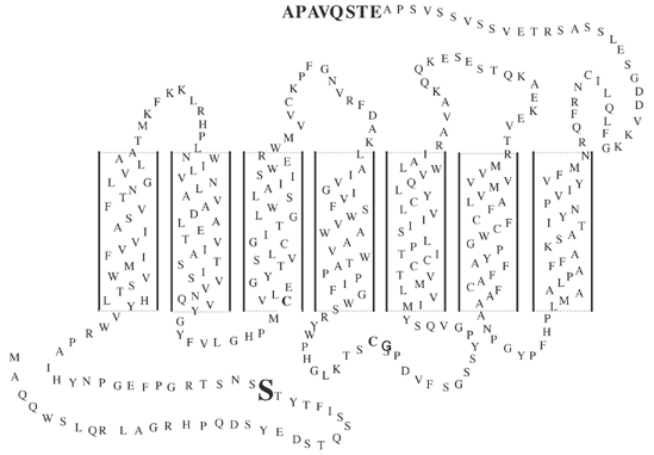

Figure 1. A secondary structure model of green cone opsin. The location of the N32S mutation is highlighted. The last eight amino acid residues shown in bold are the 1D4 epitope, which was added by in vitro mutagenesis.

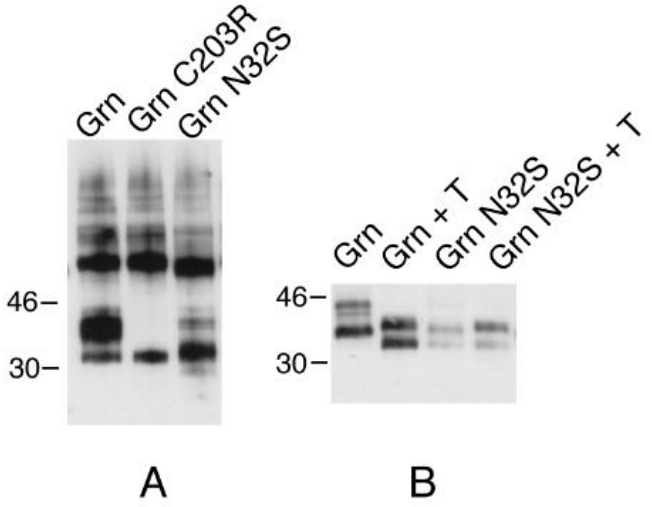

Figure 2. A Western blot analysis of wild type and mutant green opsins. Polypeptides from lysates of equal numbers of cells were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with 1D4 mAb. Immunoreactive bands were visualized using chemiluminescence (ECL) [12]. (A) The band that migrated just above the 30 kD molecular weight marker in the Grn-N32S lane represents the unglycosylated form. The band just below the 46 kd molecular weight maker in the Grn lane represents the fully glycosylated form. All bands above 46 kd represent multimeric forms of the protein. (B) Treatment with tunicamycin, Cells treated ± tunicamycin during the 16 hour induction with $CdCl_2$. Although a minor band in the Grn-N32S lane was observed to co-migrate with the glycosylated form of green opsin, digestion of the opsin with endoglycosidases N and F did not eliminate this band, suggesting that it represented another form of post-translational processing.

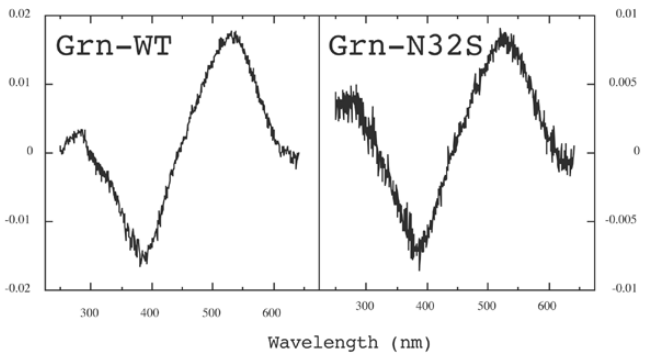

Figure 3. Photobleaching difference spectra for purified wild-type and mutant green opsins. Spectra were measured both prior to and following bleaching with light greater than 495 nm wavelength and differences were calculated. Difference spectra for wild type green (Grn-WT). Difference spectra for the N32S unglycosylated green cone opsin (Grn-N32S). Both show a λ_{max} at 530 nm.

the unglycosylated form is a less efficient pigment, perhaps due to less efficient folding. However, the function of the glycosylated and unglycosylated visual pigments were tested further by the GTPγ (^{35}S) binding assay, which showed that both the wild-type and N32S green opsins activated bovine transducin to a comparable degree in response to light (Figure 4). Using least squares regression analysis, the slopes (± standard errors) are: Grn-WT 296.7±19.3, Grn-N32S 301.4±35.9, pMEP4β12.3±1.9. The test statistic (two sample t-test) for the difference in slopes between Grn-WT and Grn-N32S was 0.12 (p=0.91). The test statistic for the difference in slopes between Grn-WT and pMEP4βwas 14.9 (p<0.0001) and between Grn-N32S and pMEP4β was 8.0 (p<0.001).

The green opsin visual pigment is not palmitoylated: To determine whether the green opsin visual pigment was palmitoylated, the epitope-tagged, wild-type green opsin, rhodopsin and mutant (C187R) rhodopsin were expressed in 293-EBNA cells in the presence of ^{3}H-palmitic acid, immunoprecipitated using the 1D4 antibody and analyzed by polyacrylamide gel electrophoresis. A band was observed for rhodopsin, but not the green cone opsin, nor for the rhodopsin mutant C187R, a polypeptide that is known to be aberrantly folded and not palmitoylated (Figure 5). These findings were confirmed by direct chemical analysis of the fatty acids extracted from wild type rhodopsin and green opsin (Table 1, p =.035).

DISCUSSION

These studies demonstrate that glycosylation is not required for the X-linked cone opsins to form a visual pigment. Because the green cone opsin is a membrane-bound protein, glycosylation of the protein occurs at its N-linked site during its passage through the endoplasmic reticulum, regardless of any functional requirements. Inhibition of rhodopsin glycosylation by treatment with tunicamycin did not affect in vivo folding, assembly, or transport of the opsin in COS-1 cells, nor the formation of a visual pigment with 11-*cis*-retinal; however, the unglycosylated rhodopsin was only 10% as efficient at achieving light-dependent activation of transducin at comparable concentration levels of the visual pigment. Of the two sites at which N-linked glycosylation occurs (N2 and N15), only N15 glycosylation appeared to be important for maximally efficient activation of transducin [17]. The absence of glycosylation at this site did not affect the formation of the MII species of rhodopsin, nor the stability of the retinyl-opsin linkage. Rather the lower stability of the unglycosylated MII intermediate compared to the glycosylated form appeared to account for these differences in transducin activation [17]. Likewise, unglycosylation of the β_2-adrenergic receptor impaired agonist-dependent signal transduction [24]. The green cone opsin differs from these other 7-transmembranous segment receptor proteins by not requiring glycosylation for signal transduction; however, the possibility cannot be excluded that unglycosylation of the cone opsin might have subtle effects on the stability of the protein that were not tested in this series of experiments.

These studies also demonstrate that, unlike rhodopsin, the X-linked cone opsins are not palmitoylated. Palmitoylation of polypeptides usually occurs at the consensus site of paired cysteine residues, sites that are present in rhodopsin (C322 and C323) and absent in the cone opsins. However, for some proteins, such as the β_2-adrenergic receptor, palmitoylation

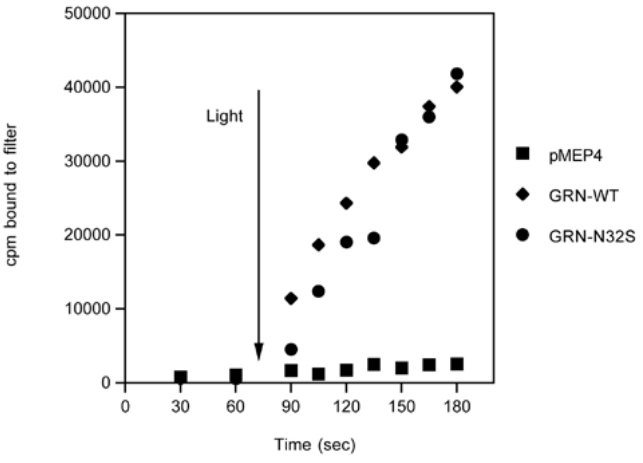

Figure 4. Light-dependent transducin activation by wild-type green opsin and the N32S unglycosylated green cone opsin. Following induction of pGrn-WT and pGrn-N32S, membrane preparations were made, then tested for their ability to bind γ-S-GTP in the presence of bovine rod transducin. PMEP4β is the cell line that was transfected with the non-recombinant vector (no opsin cDNA insert) and serves as a control for these studies.

Figure 5. Immunoprecipitation of rhodopsin and green cone opsin labeled with ^{3}H-palmitic acid. Equal volumes of immunoprecipitated material were analyzed by 12% SDS-PAGE and visualized by autoradiography.

TABLE 1. ANALYSIS OF PALMITIC ACID CONTENT IN RHODOPSIN AND GREEN CONE OPSIN

Sample	Protein (µg)	Palmitic Acid (PA) (µg)	PA/Protein	SEM
Rho	29.0	0.16	0.0055	0.0003
Grn	24.4	0.03	0.0012	0.00006

occurs at a single cysteine [25]. Although a cysteine is found at residue 332 in the cone opsin molecule, this amino acid is apparently not used for palmitoylation.

For rhodopsin, the paired palmitic acid residues are inserted in the plasma membrane, anchoring the C-terminal tail and creating a fourth cytoplasmic loop [19,20]. The absence of the cysteines suggests that the cone opsin does not have a comparable transmembranous region and thus is likely to have a different structure in its cytoplasmic C-terminal region. Differences in the palmitoylation and structure of the opsins could affect their physical properties. Receptor palmitoylation has been linked to desensitization, a process that includes phosphorylation and binding arrestin, an inhibitory protein, following exposure to the agonist or stimulus [26]. β_2-adrenergic receptors in which the palmitoylation site has been mutated are more phosphorylated and less active in signaling than wild-type. Continued agonist exposure causes uncoupling of the wild type, but not the mutant, receptor to the G-binding protein. These findings suggest that the unpalmitoylated mutant protein is desensitized compared to the wild type [25].

A comparable link between photoreceptor desensitization and palmitoylation has not been demonstrated for rhodopsin. Rhodopsin mutants that were not palmitoylated did not to show either a decrease in transducin activation nor an increase in light-dependent phosphorylation [19]. By contrast, removal of palmitate from rhodopsin in the rod outer segment membranes by treatment with hydroxylamine led to an increase in light-dependent transducin binding, despite a reduction in visual pigment regeneration with the chromophore [27]. None of these studies demonstrates a link between photoreceptor desensitization and palmitoylation; however, the role of palmitoylation has not been tested for the other important aspect of photoreceptor desensitization, arrestin binding. Hence, the significance of palmitoylation for rhodopsin function remains undetermined.

Previously we have shown that the common mutation associated with color vision deficiencies, C203R, is unglycosylated, aberrantly folded and deficient in transport to the cell membrane [15]. The absence of glycosylation of this protein appears to be a consequence of the aberrant folding. Because glycosylation appears to have no effects on cone opsin visual pigment formation, the aberrant folding and transport are sufficient to explain the association of this mutation with color vision deficiencies [13,14].

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The typographical corrections below were made to the article on the date noted. These changes have been incorporated in the article and the details are documented here.

13 December 1998: In the fourth (last) paragraph of the Methods section, "2/degrees/minute" was changed to "2 °C/min".
17 December 1998: In Table 1, "0.016" was changed to "0.16" µg palmitic acid for rhodopsin. In the Abstract, second paragraph of Methods, first paragraph of Results and Figure 2 caption, "Western" was changed to "western."