Molecular Cloning of the Salamander Red and Blue Cone Visual Pigments

Lin Xu, E. Starr Hazard III, D. Kashelle Lockman, Rosalie K. Crouch, and Jian-xing Ma

Department of Ophthalmology, Medical University of South Carolina, Charleston, SC

Purpose: Salamander retinas are known to contain at least three cone pigments and two rod pigments. The purpose of this study was to clone and characterize the visual pigments from salamander cones.

Methods: cDNA fragments of cone pigments were amplified from a salamander retina cDNA library by PCR using a pair of primers with consensus for visual pigments. These fragments were cloned and used as probes for library-screening. The full-length cDNAs were isolated from the retinal library using the cloned PCR products as probes. DNA sequences were determined by the dideoxynucleotide chain termination method.

Results: Two pigment cDNAs were cloned and sequenced from the salamander library. The global GenBank search showed that they do not match any existing sequences but have significant sequence similarity to visual pigments. One of the pigment cDNAs showed a high sequence homology with red cone pigments from other species and thus, was designated as a red cone opsin. The other pigment was designated as a blue cone opsin as it is most homologous to the chicken and goldfish blue cone pigments. Both cDNAs contain a full-length coding region encoding 365 amino acids in the red and 363 amino acids in the blue cone pigment. Hydropathy analysis predicted that both pigments could form seven hydrophobic transmembrane helices. Both pigments retain the key amino acid residues critical for maintaining the structure and function of opsins and have similar G-protein interaction sequences which differ from that of rod opsin. Phylogenetic analysis indicates that the red opsin belongs to the L group and the blue opsin belongs to the M1 group of visual pigments.

Conclusions: The salamander red and blue cone pigments share high sequence homology with the cone pigments of other species.

Most vertebrates have two types of photoreceptor cells, rods and cones in the retina. Rods are responsible for dim light vision while cones are responsible for color vision [1,2]. In most vertebrates, there is only one rod but 2-4 types of cone photoreceptor cells containing different cone pigments [3]. Vision is mediated by visual pigments located in the outer segment membranes of photoreceptor cells. Visual pigments are light-absorbing molecules and consist of a covalent complex of an apoprotein, opsin, and a chromophore, 11-cis retinal or a retinal derivative [2,4]. Upon photoactivation, visual pigments change their conformation and consequently activate G-proteins [2].

Amphibian photoreceptor cells are used extensively for vision research; some of the first observations of visual pigments were made on amphibian retinas [5-7]. Salamander photoreceptors have been extensively used for vision research because they are large in size and easy to isolate, providing a favorable model for physiological experiments [8]. Many spectroscopic, biochemical and electrophysiological studies on phototransduction have been conducted using the salamander photoreceptor cells [8,9]. Spectroscopic and electrophysiological measurements indicate that there are two types of rod cells and at least three types of cone cells in the salamander retina [9-11]. The three types of cone cells show different maximal sensitivity at wavelengths 610 nm (red cone), 444 nm (blue cone), and below 400 nm (ultraviolet cone) [10]. Interestingly, Makino & Dodd have reported that there are multiple pigments within a single salamander cone cell [11]. We have previously reported the cloning of a rod opsin from tiger salamander retina [12], but none of the salamander cone pigment sequences have been previously reported.

We report here the cloning and sequencing of two cone pigments from salamander retina. One sequence is closely related to red cone visual pigments, whereas the other has high sequence homology to blue cone pigments.

METHODS

Polymerase Chain Reaction (PCR)— To obtain a probe for library screening, PCR was performed using DNA of a salamander retina cDNA library (a gift from Dr. J. L. Arriza) as the template. Moderately degenerate PCR primers were designed based on the consensus sequences of conserved regions of known visual pigments. The primers were synthesized by a DNA synthesizer at the Medical University of South Carolina. The PCR mixture was composed of 0.1 µg of the library DNA, 50 pmol of each primer (5’ primer: 5'-TAGATGA[T/C][A/G]GG[A/G]TTGTAGAT-3’ and 3’ primer: 5'-AACT[G/A]CATCCTG[C/G][T/C/G][AACCTG-3’), 0.25 mM dNTP, 5 µl of 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin), and 2.5 U of AmpliTaq DNA polymerase in a total volume of 50 µl. PCR was carried out for 30 cycles of 94°C for one min, 50°C for two min, and 72°C for three min in a Robocycler (Stratagene, La Jolla, CA).

Southern Blot Analysis and Colony Hybridization— The
PCR products were subjected to Southern blot analysis using a nested oligonucleotide probe (5'-[C/ T][GGCTACTCTCGT(C/T)][CT/G][GGC-3']) consensus to cone pigments. The identified PCR products were purified by agarose gel electrophoresis and cloned into the pCR<sup>TM</sup> II vector (TA cloning kit, Invitrogen, San Diego, CA) which was then introduced into an <i>E. coli</i> strain Top10 cells. Positive colonies were identified by the same oligonucleotide probe by colony hybridization as described [12]. The sequences of the inserts were determined and compared with existing sequences in GenBank. The inserts of novel sequences were released and used as probes for screening the cDNA library of salamander retina.

**Screening of cDNA Library of Salamander Retina**—The salamander retina cDNA library was constructed in the λZAPII phage vector (Stratagene). Screening of the library was carried out according to the standard plaque-lifting protocol [13]. Plaques were transferred to S & S (Schleicher & Schuell, Keene NH) nitrocellulose membranes. The clones were plaque-purified by secondary and tertiary screening. For library screening. The resultant positive phage clones were plaque-purified by secondary and tertiary screening. These clones were converted to pBluescript II by the nick-translation method using 32P by the nick-translation method and used as probes for library screening. The DNA sequences were processed with the GCG implementation of PAUP 4* (Phylogeny program and aligned using the GCG program PILEUP.

**DNA Sequencing**—Double-stranded plasmid DNA was isolated from the positive clones according to Sambrook et al. [13]. Inserts were sequenced on both strands by Sanger’s dideoxynucleotide chain termination method using dsDNA Cycling Sequencing System (GIBCO/BRL) [14]. All sequences were confirmed by sequencing the complementary strand and verified in at least three independent clones. DNA sequence analysis was conducted using the Genetics Computer Group’s (GCC; Madison, WI) Wisconsin Package for Unix Systems. The amino acid sequence was deduced using the GCC TRANSLATE program.

**Phylogenetic Analysis**—The DNA sequences were retrieved, assembled to cDNA using codon notations of the submitting authors with the GCG ASSEMBLE program and aligned using the GCG program PILEUP.

The resulting multiple sequence file was passed to the GCG implementation of PAUP 4* (Phylogeny Analysis Using Parsimony) for UNIX [15]. Both the nucleotide and peptide sequences were processed with

---

**Figure 1A.** Nucleotide and deduced amino acid sequences of the salamander red and blue cone pigments. The nucleotide sequences is shown on the lower lines with the deduced amino acid sequence on the upper lines. The amino acid sequences are numbered with the first Met as +1. The termination codons are indicated by an asterisk (*). (A) Salamander red cone pigment (Genbank accession number AF038947); (B) Salamander blue cone pigment (Genbank accession number AF038946).  

**Figure 1B.**
the PAUP heuristic search bootstrap option. One hundred bootstrap samples (the default for PAUP 4*) were collected and a majority rule consensus tree was calculated. The original PILEUP alignments were also analyzed by CLUSTALW v1.73 [16]. The CLUSTALW alignments were processed by the PHYLIP programs SEQBOOT, DNADISTANCE, FITCH, and CONSENSE to produce majority rule consensus trees from the bootstrap process [17]. The CLUSTALW alignments were also submitted to the PAUP 4* program with the heuristic search and bootstrap options. The consensus trees were loaded into TreeView (v1.5) and printed as unrooted trees [18]. The L, M1, M2, and S [19] labels were added along with the supporting bootstrap replicate numbers by MacDraw. The DNA distance phylogram includes a calibration mark indicating 10 percent difference in sequence homology. The unrooted presentations were chosen to plot the trees because we are assigning molecular similarity in the present paper rather than addressing the evolution of vertebrate opsins per se.

RESULTS

Cloning and Sequence Analysis of the Cone Visual Pigments—PCR products of the expected length (700 bp) were amplified from the salamander retina library. After cloning and sequencing, two types of colonies harboring distinct sequences were obtained. Both of the sequences were found to be similar to the visual pigments: one had the highest sequence similarity to the red cone opsins from several species and the other is more similar to the blue cone opsins of chicken and goldfish. These inserts were isolated and used as probes to screen the cDNA library. A total of 26 independent clones of red cone opsin and four clones of the blue cone opsin were isolated from 10^6 pfu (plaque forming units). Sequence analysis showed the cDNA of the red cone pigment to be 1617 bp, consisting of a full-length coding region of 1095 bp, 151 bp of 5’ untranslated region (UTR), and 371 bp of 3’ UTR (Figure 1A). The first ATG (nucleotide 152), in a favorable context of Kozak sequence [20] for translation initiation with G at position -3 was designated as the translation initiation codon, and the stop codon TAG were identified at position 1247.

The blue cone pigment is 1479 bp, consisting of a full-length coding region of 1089 bp, 172 bp of 5’ UTR, and 218 bp of 3’ UTR (Figure 1B). The translation initiation codon ATG, in a context of Kozak sequence with a G at position -3, and termination codon TAG were identified at positions 173 and 1262, respectively.

Deduced Amino Acid Sequence and Predicted Secondary Structure—The red and blue cone opsin cDNAs encode 365 and 363 amino acids, respectively. The deduced proteins were predicted to have molecular weights of 40,991 for the red and 40,313 for the blue cone pigment. The hydropathy analysis of these sequences suggested that they could form seven transmembrane helices [21]. Based on the sequence similarity to bovine rhodopsin, the putative secondary structure of these visual pigments were predicted as shown in Figure 2. Based on the consensus sequence Asn-X-Ser/Thr, two glycosylation sites were identified at Asn33 and Asn53 in the red cone opsin (Figure 2A) and at Asn26 and Asn209 in the blue opsin (Figure 2B). The chromophore attachment site is conserved in the seventh transmembrane helix, Lys311 in the red and Lys305 in the blue cone pigment. These cone opsins also contain the Schiff’s base counterion, Glu128 in the red and Glu122 in the

---

Figure 2. Predicted secondary structures of the red and blue cone pigments. The transmembrane domains (boxed) were defined based on Kyte-Doolittle hydropathy plots and the comparison with bovine rhodopsin. The Lys residues at chromophore attachment sites and the Schiff’s base counterion Glu residues are indicated by diamond boxes and numbering their positions. The ERY motif is shaded. The potential glycosylation sites are indicated by an asterisk (*). Residues identical to the salamander rhodopsin are in black. Figure 2A. Predicted secondary structure of the red cone pigment. Figure 2B. Predicted secondary structure of the blue cone pigment.
blue cone pigment. The two Cys residues which form a disulfide bond in the visual pigments are also retained in these two opsins as indicated in Figure 2.

Sequence Comparison with Other Visual Pigments—

Neither of the cone opsins matches any existing sequences in GenBank (release 104.0, December, 1997). Sequence analysis shows that both cone opsins have significant sequence similarity with rhodopsin and cone visual pigments of a number of other species. At the nucleotide level, red cone opsin has 73-80% homology with red cone pigments from other species and 81-88% at the amino acid level. The highest sequence similarity was found between salamander red cone and other amphibian red cones such as Xenopus red pigment (80% at the nucleotide level and 88% at the amino acid level). The blue cone opsin has highest sequence homology to the short wavelength cone opsins. At the amino acid level, the homology is 73% to chicken blue, 69% to goldfish blue, 51.8% to chicken violet, 51.2% to human blue, and 48.2% to bovine blue cone opsin. The red and blue cone opsins share significant sequence homology to each other, with 58.5% and 42% homology at the nucleotide and amino acid levels, respectively. They also have significant sequence homology to the salamander rhodopsin [12]. The red cone opsin is 41.6% and the blue cone opsin 53.8% identical to salamander rod opsin at the amino acid level.

**DISCUSSION**

Previous spectral studies on isolated photoreceptor cells have shown that tiger salamander retina contains two rod pigments and three cone pigments based on single cell absorbance measurements [9]. In the present study, we have cloned two salamander cone pigments and determined their sequences. The sequence comparison suggests that one codes for the red cone pigment and the other for the blue cone visual pigment.

Vertebrate visual pigments can be classified into four groups: long (L), two middle (M1 and M2), and short (S) wavelength pigments based on their absorbance spectra [3]. The phylogenetic analyses herein differ in their treatment of the chicken pineal opsin and the ancient salmon opsin (Figure 3). The branch order of the ancient salmon opsin/chicken pineal opsin is not well defined relative to the other sequences. The branch order within the M2 group of the cavefish and goldfish green pigments is variable as well. The placement of these wavelength cone opsins is one support for the evolution of rod pigments as derived from cone pigments [26,27]. However, excellent support (100 percent of the bootstrap trials) for both DNA and Protein Parsimony as well as DNA distance (with Kimura two parameter or Maximum Likelihood distance formulas), and DNA Maximum Likelihood analysis (not bootstrapped) place the salamander red cone pigment into the L group and the salamander rhodopsin into the M2 group. Blue cone opsin sequences are less conserved across species than rhodopsins and red cone opsins. As shown in the phylogenetic tree, the salamander blue cone, clustered with the chicken blue, goldfish blue, and cavefish blue are classified in the M1 group.

Figure 3. Phylogenetic analysis of salamander visual pigments. A DNA distance phylogenetic tree of visual pigments was constructed as described in Methods. The pseudonym names are presented as following: rhod, rhodopsin; red, red cone pigment; grn, green cone pigment; blue, blue cone pigment; Salmon Anct, ancient salmon opsin; prhop, pineal opsin; Viol, violet cone pigment. The GenBank accession numbers are: bovine blue [19], U92557; bovine red [34], L09725; bovine rhod [35], K00502, K00503, K00504, K00505, K00506; cavefish blue [36], S66818, S66819, S66828, S66829, S66838; cavefish grn4 [37], U12024; cavefish grn5 [37], U12025; cavefish red [38], M90075; chick blue [33], M92037; chick grn1 [33], M92038; chick prhop [39], U87449; chick red1 [30], M62903; chick red2 [40], X57490; chick rhod [41], D00702; chick viol [33], M92037; domicrat red1 [42], AF031532; goldfish blue [43], L11864; goldfish grn1 [43], L11865; goldfish grn2 [43], L11866; goldfish red1 [43], L11867; goldfish rhod [43], L11863; human blue [44], M13295, M13296, M13297, M13298, M13299; human grn [44], M13306; human red1 [44], M13300, M13301, M13302, M13303, M13304, M13305; human rhod [45], K02281; lamprey rhod [46], U67123, U67124, U67125, U67126, U67127; mouse blue [19], U49720; mouse rhod [47], M55171; salmonate blue, AF038946; salmonate red, AF038947; salmonate rhod [12], U36657; Salmon Anct, AF001499 [48].
While the human, bovine, and mouse blue pigments are in the S group (Figure 3).

Based on sequence homology, our phylogenetic analysis has made an assignment of the red and blue cone pigment to the L and M1 groups, respectively. The blue cone pigment shares 68% amino acid sequence homology to goldfish blue pigment (an M1 pigment) and 48% to goldfish UV cone pigment (an S pigment). Recently, we have cloned a salamander S-group cone pigment which was confirmed to be a UV pigment by spectral analysis (data not shown). This UV pigment is 48.7% identical to goldfish blue and 62% to goldfish UV cone pigment. Therefore, the salamander blue pigment sequence is more similar to the M1 blue pigments than to the S group blue/UV pigments. Based on these observations, we classify the salamander blue pigment as an M1 pigment. This assignment remains to be confirmed by expression and spectral analysis.

In bovine rhodopsin, the C-terminus has a Cys pair. The blue cone opsin contains only one Cys residue (Cys332), while the red one lacks Cys residues in the corresponding region, suggesting that the red cone pigment may have a different C-terminal domain compared to rhodopsin. Sequence alignment of visual pigments revealed that this is consistent with visual pigments from other species: M2 group pigments including rhodopsins have the Cys pair. All of the blue pigments (in M1 and S group) have a single Cys, while L group cone pigments have no Cys residues. The Cys pair in the C-terminus of bovine rhodopsin is known to be attached by palmitoyl groups which anchor to the membrane to form an extra cytoplasmic loop [28,29]. It remains to be determined whether the single Cys in the C-terminal region of the blue cone pigment is palmitoylated.

The C-terminal region of visual pigments is known to be Ser and Thr residue-rich. These residues serve as phosphorylation sites [30]. Among the 25 C-terminal residues in the visual pigments, there are 11 Ser/Thr residues in the red cone pigment and 10 in the blue pigment. Similarly, the salamander rhodopsin cloned previously contains 12 Ser/Thr among the 25 C-terminal amino acid residues. The C-terminal sequences of the red and blue cone visual pigments have a higher degree of sequence homology to the C-terminal region of rhodopsin (54% and blue 60% identical to rhodopsin). As the C-terminal region is known to contain the phosphorylation sites, high sequence homology at this region suggests that these cone pigments may undergo a similar phosphorylation pathway to rhodopsin’s.

Salamander retina is known to be rod dominant [10,31]. Among the three types of cone photoreceptors, the red cone cells are more prevalent than the blue and UV cones [10]. In the screening of the salamander retina cDNA library, we have identified 26 clones of red, four clones of blue and three clones of UV cone opsins from 10^6 pfu, suggesting that red cone pigment is more abundant than the blue and UV pigments. This is consistent with a higher prevalence of red cone cells [10,31]. This is also true for other species, for example, chicken iodopsin is the most abundant cone pigment [32,33].

The sequence comparison demonstrates that salamander red and blue cone pigments share high sequence homology to other species including their mammalian counterparts, suggesting that salamander cones are a valid model for vision research.

ACKNOWLEDGEMENTS
The authors would like to thank Dr. J. L. Arriza in the Volum Institute of Oregon Health Science University for kindly providing the salamander retina cDNA library and Dr. D. W. Corson for helpful discussions. The GCG Unix System software was provided by Biomolecular Computing Resources (BCR) at the Medical University of South Carolina. This work was supported by grants from the National Science Foundation, MCB96-00772 and EPS-9630167 (J. Ma) and a grant from National Institutes of Health, EY04939 (R. K. Crouch).

REFERENCES