Molecular and Biochemical Analyses of Iodopsin in rd Chick Retina

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**Purpose.** The results of previous immunocytochemical and electrophysiological studies of retinas of rd (retinal degeneration) chicks suggest that the iodopsin cone visual pigment may be defective in this mutant. The goal of this study was to determine if the primary structure and synthesis of this protein is normal in this animal model of inherited retinal degeneration.

**Methods.** Northern cDNA sequence and western analyses were used to study rd/rd iodopsin. cDNAs encoding rd/rd iodopsin were obtained by screening an rd/rd cDNA retinal expression library and by reverse transcription PCR. Western blots were probed with either R4 or COS-1, two different monoclonal antibodies that have been shown to specifically recognize chicken iodopsin.

**Results.** Hybridization of the +/+ , +/rd, and rd/rd poly(A)+ RNA with an iodopsin cDNA probe revealed the presence of a single 1.5 kb band in each of the samples, all of which were labeled with equal intensity. No significant differences were found between the published nucleic acid sequences for normal chicken iodopsin cDNA and that determined for rd/rd iodopsin cDNA. Antibody-dependent differences in the staining intensity of the 34 kDa band containing iodopsin were observed on western blots of +/+ , +/rd, and rd/rd retinal protein. R4 stained the 34 kDa band in each sample with equal intensity. COS-1 labeling of the 34 kDa band in the rd/rd sample was less intense than that observed in the +/+ and +/rd samples.

**Conclusions.** Based on the results of the cDNA sequence and northern blot experiments, the authors conclude that the gene encoding iodopsin and transcription of this gene are normal in the rd mutant. The results of the western blot analyses of rd/rd iodopsin suggest that post-translational processing of iodopsin may be abnormal in this mutant. Invest Ophthalmol Vis Sci. 1994;35:2550-2557.
protein moiety. The red-sensitive cone pigment, iodopsin, and the rod pigment, rhodopsin, account for approximately 89% of the total pigment found in chick retina. The possibility that either of these pigments could be defective in the rd mutant was first suggested by the observation that the a-wave of the ERG recorded from rd/rd retina is severely attenuated or absent. Since then, immunocytochemical, biochemical, and electrophysiological techniques have been used to examine rhodopsin and iodopsin in rd chick retina.

The results of immunocytochemical and biochemical studies of rd/rd retina suggest that rhodopsin is normal in this mutant. Using a monoclonal antibody specific to the C-terminal region of bovine rhodopsin (K16-155), Ulshafer et al found that immunolabeling patterns and quantities of rhodopsin in rd/rd retina were virtually indistinguishable from those seen in +/+ retina, a result that suggests that the amount and structure of rhodopsin protein is normal in the rd mutant. Their findings were consistent with the results of previous biochemical studies of retinyl palmitate-stearate, unesterified retinol (both 11-cis and all-trans isomers) and interstitial retinol-binding protein in rd/rd retina that suggested that the vitamin A cycle and rhodopsin are normal in the rd mutant.

Two lines of evidence suggest that the cone visual pigment, iodopsin, may be abnormal in the rd chick. Immunocytochemical studies using a monoclonal antibody specific to middle-to-long wavelength sensitive cones (COS-1) showed that although the number of cones stained in rd/rd retina was the same as observed in +/+ retina before the onset of photoreceptor degeneration, the density of staining of the cone outer segments in rd/rd retina was significantly less (P < 0.001) than that of the +/rd cone outer segments. These data suggest either that less iodopsin pigment is present in rd/rd cone photoreceptors or that the structure of the iodopsin protein is altered in the mutant so that the antibody binds to its epitope less efficiently. Analyses of rd/rd early receptor potentials (ERPs) provided a second measure of cone visual pigment integrity in this retina. The ERP is generated by the redistribution of charge on pigment molecules as they undergo conformational changes after absorption of light. The amplitude of the ERP is linearly proportional to the number of pigment molecules activated by the light stimulus and in retinas containing both rod and cone photoreceptors, such as that found in chick, the ERP response is dominated by the cone cells. Measurements of the ERP in rd/rd retina revealed that this response was either absent from, or severely attenuated in, chicks 1 to 3 days after hatch. These results are consistent with the immunocytochemical data in suggesting that either the amount or the structure of iodopsin may be abnormal in rd/rd retina.

In the present series of experiments, northern, DNA sequence and western analyses were carried out on iodopsin mRNA, cDNA, and protein, respectively, to determine if the primary structure, transcription, and translation of the iodopsin gene is normal in the rd chick mutant.

MATERIALS AND METHODS

Northern Blot Analyses

Poly(A)^+ RNA was prepared from +/+, +/rd, and rd/rd retina-pigment epithelium-choroid using a Fast-Track RNA isolation kit (Invitrogen, San Diego, CA.). All animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research and the guidelines established by the Institutional Animal Care and Use Committee at the University of Florida. The samples, each containing approximately 2.5 μg mRNA and 5 μg of a 0.24 to 9.5 kb RNA ladder (BRL), were electrophoresed in a 1% agarose gel containing 1.1% formaldehyde, 20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate, pH 7.0. After electrophoresis, the gel was rinsed 2 X 20 minutes in 10 X SSC and transferred by capillary action to nylon membrane (Biotrans, ICN, Costa Mesa, CA) overnight at room temperature. After transfer, the RNA was UV-crosslinked to the membrane (UV Stratalinkrer, Stratagene, La Jolla, CA) and then stained with 0.04% methylene blue in 0.5 M sodium acetate, pH 5.2, to determine the efficiency of RNA transfer. Finally, the blot was dried by baking at 65°C for 1 hour.

32P-labeled iodopsin and chick β-actin cDNA probes were synthesized from a 730 bp BamHI-PstI fragment (see Fig. 2) of iodopsin cDNA clone 20127 (clone was a gift from Dr. Fumio Tokunaga) and the PstI fragment of a chick β-actin cDNA clone (clone was a gift from Dr. Steven Pittler), respectively, using a Prime-It II random primers DNA labeling kit (Stratagene). Unincorporated nucleotides were removed from the probes using NucTrap push columns (Stratagene). Prehybridization, hybridization, and washing conditions were modified after those described by Church and Gilbert. Briefly, the prehybridization solution contained 0.5 mM EDTA, 0.5 M NaHPO4 (pH 7.4), and 7% SDS. The wash solution contained 1 mM EDTA, 40 mM NaHPO4 (pH 7.4), and 0.1% SDS. All prehybridization, hybridization, and washing steps were carried out at 60°C in a Hybaid Mini-Hybridization Oven (National Labnet, Woodbridge, NJ). For each probe, the blot was prehybridized for at least 1 hour, followed by overnight hybridization with the probe. The probes were diluted to 10^6 cpm incorporated 32P per ml hybridization solution before use. After hybridization, the blot was washed 4 X 15 minutes, wrapped in plastic wrap, and exposed to Kodak...
Restriction endonuclease maps of the clones library, we were unable to obtain a from the rd/rd confirmed that the cDNAs encoded iodopsin. Al-
cDNA was cloned using coupled reverse transcription polymerase chain reactions (RT-PCR).

Cloning and Sequencing rd/rd Iodopsin cDNA

Embryonic day 19 rd/rd chick retina-pigment epithelium-choroid poly(A)+ RNA was used to construct a cDNA library in the Uni-ZAP XR unidirectional cloning vector (Stratagene). The library, containing approximately 2.5 × 10⁶ independent recombinants, was amplified in the Escherichia coli strain PLK-F before use. Approximately 500,000 plaque-forming units of the cDNA library were plated (50,000/plate), and duplicate filters of each were prepared using 0.45 µm Immobilon NC filters (Millipore, Bedford, MA). After absorption of the phagemids to the filters, the filters were floated sequentially on three different solutions containing either 0.5 M NaOH, 1 M Tris-HCl (pH 7.5), or 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl for 2, 5, and minutes, respectively. The processed filters were then baked for 2 hours at 65°C. Both sets of filters were prehybridized in a solution containing 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10× Denhardt’s solution, 20 µg/ml heat-denatured salmon sperm DNA, and 1% SDS for 2 hours at 42°C. The random primer labeled iodopsin cDNA probe was synthesized as described above using the 1.0 kb EcoRI-PstI fragment of iodopsin clone 201 (see Fig. 2). Filters were hybridized in a solution identical to the prehybridization solution except that it contained 1X Denhardt’s solution. Denatured probe was added to the hybridization solution to a final concentration of 10⁶ cpm incorporated ³²P per ml and the filters were hybridized overnight at 42°C in a Mini-Hybird (National Labnet) hybridization oven. After hybridization, the filters were rinsed in wash solution containing 0.1 × SSC and 0.1% SDS and then washed in fresh wash solution for an additional 30 minutes at 50°C. Positive plaques were identified by exposing the filters to Kodak XAR-5 film overnight at −70°C. In vivo excision and rescue of double-stranded recombinant pBluescript SK− plasmids was performed as described by the manufacturer (Stratagene). Restriction endonuclease maps of the clones confirmed that the cDNAs encoded iodopsin. Although we isolated several clones encoding iodopsin from the rd/rd library, we were unable to obtain a clone that included the extreme 5’ end of the open reading frame. This portion of the rd/rd iodopsin cDNA was cloned using coupled reverse transcription polymerase chain reactions (RT-PCR).

RT-PCR was carried out with primers complementary to the published 5’ noncoding region of iodopsin cDNAand to a region approximately midway through the open reading frame (5’-CGAAGCTTACACGGGCGATGG-3’ and 5’-CGCTCGAGGCCAGCCGACGACCACAG-3’). A GC clamp and HindIII and XhoI sites were incorporated into the 5’ ends of the primers to facilitate cloning; however, this cloning strategy proved inefficient, and the PCR products were cloned using a ddT-tailed pBluescript SK vector. The RT-PCR reactions were carried out in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) using a GeneAmp RNA PCR kit (Perkin Elmer) according to the manufacturer’s instructions. Briefly, first-strand cDNA was synthesized from 10 ng poly(A)+ rd/rd RNA using either random hexamers, oligo(dT) or the iodopsin antisense primer, and a thermal profile of 15 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C. Upon completion, components necessary for the PCR reaction were added to each tube. In the case of the random hexamer and oligo(dT) primed RT reactions, both the sense and antisense iodopsin primers were added to the reaction mixtures. Only the sense iodopsin primer was added to the RT reactions that had been primed with the antisense iodopsin primer. The thermal profile used for the PCR was 2 minutes at 95°C, 35 cycles of 1 minute at 95°C, 1 minute at 60°C, and finally a 7-minute extension at 60°C. Reactions were held at 4°C until analysis. Restriction endonuclease maps of the resulting PCR products indicated that the cDNA products encoded the 5’ end of iodopsin. The PCR products from two independent reactions were then cloned into ddT-tailed pBluescript SK plasmid vector.

The nucleotide sequence of the rd/rd iodopsin cDNA was obtained by sequencing double-stranded DNA isolated from eight partial-length cDNA clones, as well as from the cloned PCR-generated 5’ fragments from two separate PCR reactions (Fig. 2). The DNA was sequenced by the dideoxynucleotide chain termination procedure using Sequenase 2.0 (United States Biochemical, Cleveland, OH) and T3 and T7 primers. All sequence reactions were run on 6% acrylamide gels prepared using Long-Ranger 50% acrylamide stock solution (AT Biochem, Malvern, PA). Sequence compressions were resolved on 8% Long-Ranger gels containing 40% formamide. Sequence data were analyzed using GCG sequence analysis software written for the VAX.

Western Blots

Retina-pigment epithelium-choroid was dissected from the eyes of 3 day old +/+, +/rd, and rd/rd chicks and stored at −70°C until use. Tissue samples were prepared, and the resulting proteins were separated on 10% acrylamide gels according to the method of
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Laemmli32 using a Biorad (Melville, NY) Mini-Protean II gel system. To prevent aggregation of the iodopsin protein, the protein samples were not placed in a boiling water bath before loading onto the gel. After electrophoresis, the proteins were transferred to nitrocellulose (0.45 μm, Hoefer, San Francisco, CA) in buffer containing 20 mM Tris, 150 mM glycine, and 10% methanol at a constant voltage of 90 volts for 2.5 hours at 4°C. The resulting blots were blocked in Tris-buffered saline (TBS) (0.2 M NaCl, 0.002 M MgCl2 in 0.05 M Tris-HCl, pH 7.5) containing 3% bovine serum albumin (BSA) for 1.5 hours at room temperature before incubation with either the R4 or COS-1 monoclonal iodopsin antibody. The monoclonal antibodies, R433 (gift from Dr. Y. Fukada) and COS-117 (gift from Dr. P. Rohlich) were used at a dilution of 1:1000 and 1:50, respectively. Antibodies were diluted with 1% BSA in TBS, and blots were incubated in this solution overnight at 4°C. After incubation with the primary antibody, the blots were washed 3 × 15 minutes in TBS followed by a 1-hour incubation at room temperature in goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) diluted 1:500 with 1% BSA in TBS. The blots were then washed as before and the protein-antibody complexes were visualized by incubating the blots in alkaline phosphatase substrate (100 mM NaCl, 5 mM MgCl2, 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, and 0.2 mM Nitro Blue tetrazolium in 100 mM Tris-HCl, pH 9.5) for approximately 5 minutes.

RESULTS

The size of the normal iodopsin gene transcript has been reported to be 1.5 kb.29 The results of our northern blot analyses of poly(A) + RNA prepared from +/+, +/rd, and rd/rd retina showed that the 32P-labeled PstI-BamHI iodopsin probe hybridized to a single 1.5 kb band in each of the samples, a result corroborating the previous size estimate for the iodopsin transcript (Fig. 1A). Reprobing the blot with a 32P-labeled chick β-actin probe revealed that approximately equal amounts of mRNA had been loaded for each sample (Fig. 1B). Radioanalytic analyses of the blot using an AMBIS imaging system showed that the labeling intensity of the 1.5 kb iodopsin band relative to that of the 2.0 kb β-actin band was not different between samples.

The cDNA encoding the rd/rd iodopsin protein was cloned and sequenced to determine if the open reading frame contained a mutation that would alter the primary structure and possibly the function of the protein. This was accomplished by comparing the rd/rd sequence with the two published sequences for normal chick iodopsin.27,29 In the Kuwata et al29 sequence, codon 55 (CTG) and codon 240 (TCG) encode leucine and serine, respectively. In contrast, in the sequence published by Tokunaga et al,27 codon 55 (CTG) and codon 240 (TCG) encode valine and tryptophan. The entire sequence of the rd/rd iodopsin cDNA was established by combining the overlapping sequences of eight independent cDNA clones and two PCR-generated clones containing the 5’ region of the cDNA (Fig. 2). The results of our analyses showed that the entire rd/rd cDNA sequence matched the normal iodopsin sequences. In our rd/rd sequence, codon 55 (CTG) and codon 240 (TCG) were found to encode leucine and tryptophan, respectively, a result in agreement with a composite of the published normal sequences.

Finally, western blots containing total protein extracted from +/+, +/rd, and rd/rd chick retinal pigment epithelium-choroid poly(A) + RNA hybridized with (A) iodopsin and (B) β-actin cDNA probes. Lanes 1, 2, and 3 contained 2.5 μg of either +/+, +/rd, or rd/rd mRNA, respectively. Hybridization of the blot with the β-actin probe was done to control for RNA loading differences between samples. The final positions of the standard RNA markers (BRL) are indicated at the left.
FIGURE 2. Probe and sequencing strategy for the rd/rd iodopsin cDNA. The BamHI-PstI probe was used in the northern blot analyses, and the EcoRI-PstI probe was used to screen the rd/rd library for iodopsin cDNA clones. The restriction sites indicated on the drawing were used to verify the identities of the cDNA clones and to subclone portions of the cDNA for sequence analyses.

FIGURE 3. Western blot of +/+, +/rd, and rd/rd retina-pigment epithelium-choroid total protein probed with either the R4 or COS-1 monoclonal iodopsin antibody. (A) Control lane loaded with 30 µg total +/+ protein. The primary antibody was omitted from the staining procedure. The two bands noted on the blot are due to nonspecific staining of these chick proteins by the secondary antibody. (B) R4 staining of 30 µg rd/rd (lane 1), +/rd (lane 2), and +/+ (lane 3) total protein. (C) COS-1 staining of 40 µg rd/rd (lane 1), +/rd (lane 2), and +/+ (lane 3) total protein. (B and C) The arrow indicates the position of the 34 kDa band. Positions of the molecular weight markers are shown on the right.

Support for the hypothesis that the iodopsin cone pigment is abnormal in the rd chick has come from immunocytochemical and electrophysiological studies of rd/rd retina. Based on the results of these studies, it was suggested that the structure, quantity, or both of the iodopsin protein is abnormal in rd/rd retina. In the present series of experiments, we used northern, cDNA sequence, and western blot techniques to test this hypothesis.

Our sequence analyses of rd/rd iodopsin cDNA revealed that the nucleotide sequence of the open reading frame encoding rd/rd iodopsin is identical to the sequences previously reported for normal chicken iodopsin. Based on this result, we conclude that the amino acid sequence of the iodopsin protein is normal in rd/rd retina. Thus, the results of previous studies of rd/rd retina showing reduced labeling of cone outer segments by the iodopsin antibody COS-1 and of abnormally small or absent ERP responses cannot be attributed to a defect in the primary structure of iodopsin.

The results of our immunoblot experiments using the R4 antibody showed that the amounts of iodopsin present in rd/rd, +/rd, and +/+ retina are comparable. This result, which was in accord with our northern blot results, was at odds with the results of the previous

SA). Staining intensity of the 34 kDa band by the R4 antibody was comparable for the three retinal samples in all blots tested (Fig. 3B). In contrast, the staining intensity of the 34 kDa band by the COS-1 antibody decreased across samples with heaviest staining in the +/+ sample and lightest staining in the rd/rd sample (Fig. 3C). The staining intensity differences observed with COS-1 on the immunoblots were consistent with previous immunocytochemical analyses of these retinas using this antibody.18

DISCUSSION

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COS-1 immunocytochemical experiments that suggested that rd/rd cone photoreceptors contain less iodopsin pigment than +/rd cone photoreceptors.\textsuperscript{18} Two factors that could contribute to the discrepancy in these results are differences in the binding characteristics of the R4 and COS-1 antibodies and differences intrinsic to the immunocytochemical and western blot techniques used in these experiments. To resolve this discrepancy, we obtained a small quantity of COS-1 antibody and repeated the immunoblot experiments. The immunoblot results obtained with the COS-1 antibody were similar to those obtained in the previous immunocytochemical study,\textsuperscript{18} namely, COS-1 staining of the 34 kDa band in the rd/rd sample was less intense than that observed in the +/rd sample. In addition to the rd/rd and +/rd samples, we also tested +/+ retina samples and found that the amount of iodopsin staining in the +/rd sample was less than that observed in the +/+ sample.

What conclusions can be drawn from these data? Together, the R4 immunoblot and northern blot data are consistent with the conclusion that +/+, +/rd, and rd/rd retinas contain similar amounts of iodopsin. The fact that the staining of the +/rd and rd/rd 34 kDa bands by COS-1 differs from the staining observed with the R4 antibody under identical immunoblot conditions suggests that decreased COS-1 staining of the 34 kDa band in the +/rd and rd/rd samples may be due to a modification of the COS-1 epitope in these samples. Recently, Röhllich and Szé\textsuperscript{34} mapped the epitope recognized by the COS-1 antibody to the last six amino acids of the C-terminus of the cone pigment protein. As is the case for rhodopsin, this region of the protein is enriched with serine and threonine residues and has been shown in vitro to be phosphorylated by opsin kinase in a light-dependent manner.\textsuperscript{35} The epitope recognized by the R4 antibody has not been determined, but experiments carried out by Shichida et al\textsuperscript{38} suggest that it is different from that recognized by COS-1. Thus, one possible explanation for decreased staining of the 34 kDa band by COS-1 in +/rd and rd/rd retina could be that the C-terminus of the iodopsin visual pigment is abnormally phosphorylated in fewer transducin molecules because the affinity of the photoactivated pigment for the transducin would be reduced and the ability of the pigment to bind arrestin would be enhanced. This scenario would be consistent with the previous observation that the ERG responses generated by the rd retinas at hatch are essentially absent.\textsuperscript{3,5}

Abnormalities in rhodopsin phosphorylation have been reported in retinal extracts of two other animal models of inherited retinal disease: the rd mouse\textsuperscript{42} and Irish setter dogs with rod-cone dysplasia (rcdl).\textsuperscript{43,44} In rd/rd mice, absence of rhodopsin phosphorylation has recently been shown to be due to an increase in the activity level of protein phosphatase 2A.\textsuperscript{45} Protein phosphatase activity also appears to be elevated in retinas of Irish setter dogs with rod-cone dysplasia.\textsuperscript{44} The primary genetic defect in both these animal models has recently been shown to reside in the gene encoding the \(\beta\)-subunit of rod cGMP phosphodiesterase.\textsuperscript{46-48} Thus, abnormal rhodopsin phosphorylation appears to be secondary to disruption of cGMP metabolism in the retinas of these animals.

The primary genetic defect underlying retinal dysfunction in the rd chick has not yet been identified. As in the rd mouse and Irish setter, cGMP metabolism in rd chick retina also appears to be defective\textsuperscript{42}; however, because cGMP levels are abnormally low in pre-degenerate rd chick retina, a situation opposite to that found in rd mouse\textsuperscript{50} and rcdl Irish setter\textsuperscript{31-35} retina, it seems likely that the genetic defect in rd chick will differ from that found in rd mouse and rcdl Irish setter. Because phosphorylation processes and cGMP metabolism are interdependent in retina, further examination of pigment phosphorylation in rd/rd chick retina may prove important to our understanding of the rd defect in chicken.

**Key Words**

inherited, degeneration, cone photoreceptor, visual pigment, cDNA

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