An Opsin Homologue in the Retina and Pigment Epithelium

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Purpose. The aim of this project was to investigate the retinal pigment epithelium (RPE) at the molecular level by identification of novel RPE-specific cDNAs that may encode proteins of signal transduction pathways or other proteins that are expressed preferentially in the RPE.

Methods. A bovine RPE cDNA library was constructed in bacteriophage λg10 using RPE-enriched poly(A)+ RNA. The library was screened by differential hybridization to bovine RPE and kidney cDNA probes.

Results. A member of the heptahelical receptor family was identified in bovine RPE by molecular cloning. Its deduced amino acid sequence predicts a protein that has 291 amino acid residues and resembles most closely the family of visual pigments. A lysine residue, analogous to the retinaldehyde attachment site in rhodopsin, is conserved in the seventh hydrophobic segment of the novel sequence. Messenger RNA encoding the putative G protein-coupled receptor was detected by in situ hybridization in the RPE, inner nuclear layer, and specific cells of the ganglion cell layer. Immunohistochemical staining of bovine retina showed that the receptor protein is localized in Müller cells, as well as in the RPE.

Conclusions. A novel heptahelical receptor defines a distant evolutionary branch of the visual pigment tree. The selective localization of this putative receptor, its abundance in RPE and retina, and its homology to the visual pigments suggest that the function of this receptor is important in a visual process involving the RPE and Müller cells. Invest Ophthalmol Vis Sci. 1993;34:3669-3678.

The retinal pigment epithelium (RPE) lies adjacent to the photoreceptors and performs multiple functions essential to the visual process.1,2 The RPE forms a monolayer of highly differentiated cells that segregates the photoreceptors from the choroidal capillaries. It constitutes part of the blood-retina barrier by regulating the transport of ions, nutrients, and macromolecules between the choroidal blood supply and the photoreceptors.3,4 It is active in the uptake and storage of retinoids and in the secretion of basement membrane components. The polarized epithelial cells of the RPE are responsible for maintaining the photoreceptors by continually removing the shed fragments of outer segments through phagocytosis.5 The RPE performs this demanding phagocytic activity to enable each photoreceptor cell to renew its outer segment about every 10 days. Perturbation of the functions of the RPE may lead to degeneration of the photoreceptors and to other visual disorders.6 Another principal role of the RPE is to restore the chromophore 11-cis-retinal from its all-trans configuration and allow regeneration of the visual pigments. In the vertebrate visual system, regeneration of rhodopsin is dependent on the biosynthesis of 11-cis-retinal in the dark, a process that occurs in the RPE.7,8 Studies have shown that the isomerization of free all-trans-retinol to the 11-cis-conformation occurs at the alcohol, rather than the aldehyde, level of oxidation state.9 The isomerization step has been characterized in vitro by demonstration of cell-free 11-cis-retinoid biosynthesis.10 An enzymatic mechanism involving a lecithin retinol acyl transferase and an isomerohydrolase has been proposed for the endothermic reaction of converting all-trans-retinol to 11-cis-retinoids.11
Interesting questions remain concerning the vertebrate visual cycle, including how the metabolic flow of retinoids is controlled and how 11-cis-retinal is released specifically to the photoreceptor outer segments from the apical membrane of the RPE. The molecular genetics of the vertebrate visual cycle is less well-defined than is the molecular genetics of phototransduction. It is plausible that specific functions of the RPE involve proteins encoded by genes that are expressed preferentially in RPE cells. To investigate the RPE cell at the molecular level, we have attempted to identify and isolate RPE-specific cDNAs. In this study, we have identified and cloned from bovine RPE cells a putative G protein-coupled receptor. The retinal localization of this receptor, its high level of expression, and its homology to the visual pigments are salient features of this member of the photoreceptor family.

METHODS

This investigation was conducted in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Bovine RPE RNA and Construction of an RPE cDNA Library

Bovine RPE cells were isolated, and RNA was prepared within 2 hours of eye enucleation. After excision of the anterior segment and removal of the lens, vitreous, and neurosensory retina, RPE cells were isolated by enzymatic digestion with 50 U/ml of hyaluronidase and 100 U/ml of collagenase, followed by gentle scraping of the cell monolayer. The RPE cells were collected by centrifugation and homogenized immediately in 4 M guanidinium thiocyanate. Total cell RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction. Poly(A)+ RNA was isolated by binding to oligo(dT)-cellulose columns. The RNA was electrophoresed in 0.9% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose. The filter was hybridized for 24 hours at 42°C in a buffer containing 50% formamide, 5 X SSC, 50 mM NaH2PO4, pH 7.0, 0.2 X Denhardt’s solution, 0.1% SDS, 50 μg/ml of denatured salmon sperm DNA, and cDNA probe (10⁶ count/min • ml). The probes were labeled by nick translation using the di-deoxy chain termination method. Computer analyses of the sequences were carried out using MacVector 3.5 software (IBI, New Haven, CT). Similarity searches of nucleotide and peptide sequence databases were performed using the MacVector software package and the BLAST network service at the National Center for Biotechnology Information.

RNA Blot Hybridization

Total RNA from RPE cells and retina was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Other RNA was prepared from quick-frozen tissue by guanidinium thiocyanate extraction and LiCl precipitation. Poly(A)+ RNA was isolated by binding to oligo(dT)-cellulose columns. The RNA was electrophoresed in 0.9% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose. The filter was hybridized for 24 hours at 42°C in a buffer containing 50% formamide, 5 X SSC, 50 mM NaH2PO4, pH 7.0, 0.2 X Denhardt’s solution, 0.1% SDS, 50 μg/ml of denatured salmon sperm DNA, and cDNA probe (10⁶ count/min • ml). The probes were derived from cDNA clone RPE6 and human G-protein α₁₁-subunit cDNA and were labeled by nick translation. The final washing of the filter was performed in a solution containing 0.1 X SSC and 0.1% SDS at 54°C for 30 minutes. Autoradiography was accomplished by exposure to Kodak X-omat AR film (Eastman Kodak, Rochester, NY) at —80°C using an intensifying screen.

In Situ Hybridization

An amelanotic portion of bovine retina with attached choroid complex was embedded in OCT compound (Miles, Inc., Elkhart, IN), frozen slowly in liquid nitrogen, and stored at —80°C until sectioning. Retina sections of 10 μm were cut with a cryostat, thaw-mounted onto slides, and kept frozen until used for hybridization. The sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature, and then rinsed sequentially in PBS, water, and 0.1 M triethanolamine, pH 8.0. Acetylation was carried out at room temperature for 10 min-
utes in a solution containing 0.25% acetic anhydride, 0.1 M triethanolamine, and 0.9% NaCl. The tissue sections were dehydrated by rinsing stepwise with ethanol at increasing concentrations. In situ hybridization was performed using ³²P-labeled RNA probes. The antisense and sense RNA probes were synthesized and labeled in vitro using T3 and T7 RNA polymerases to transcribe the RPE6 cDNA in the pBluescript vector. Hybridization of the tissue sections was performed in a buffer containing 50% formamide, 4 X SSC, 5 X Denhardt's solution, 1% SDS, 0.25 mg/ml yeast tRNA, 10% dextran sulfate, 0.1 M DTT, 25 μg/ml poly A, and 25 μg/ml poly C. The hybridization was carried out for 3 hours at 50°C in a humidified environment. The probes were heated at 70°C for 5 to 10 minutes before adding to the sections at about 5 × 10⁵ cpm/section. After hybridization, the sections were treated with 20 μg/ml RNase A for 30 minutes at 37°C, and washed in fig/ml adding to the sections at about 5 X 10⁵ cpm/section.

**Antibody Production and Protein Immunoblot Analysis**

A 13 amino acid-long peptide (CLSPQRREHSREQ), which corresponds to the deduced carboxyl terminus of bovine RGR, a putative heptahelical receptor, was synthesized and conjugated to keyhole limpet hemocyanin. The production of antibody in rabbits was carried out by Cocalico Biologicals (Reamstown, PA). The antipeptide antibodies were affinity-purified by immunoadsorption to the cognate peptide coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). For immunoblot analysis, bovine tissues were homogenized using a Brinkmann (Westbury, NY) polycrystal at the no. 5 setting for 30 seconds in a buffer containing 10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 250 mM sucrose, and 0.2 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). For each tissue, 100 mg of total cell protein was electrophoresed in duplicate 12.5% SDS polyacrylamide gels. After electrophoresis, one gel was stained with 0.5% Coomassie blue, and the duplicate gel was submitted to electrophoretic transfer of proteins onto nitrocellulose filter. The protein blot was incubated first with the affinity-purified antipeptide antibody and then with secondary antibody, alkaline phosphatase-conjugated goat antibody against rabbit IgG. The binding assay was developed with the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Immunohistochemical Localization**

Bovine eyes were obtained postmortem from a local abattoir. After removal of the anterior segment and vitreous humor, the eye cup was immersed for 2 hours at 4°C in 4% paraformaldehyde in PBS. The tissue was then infiltrated overnight with cryoprotectant 30% sucrose in PBS. The central tapetal region of the retina containing amelanotic RPE cells was dissected from the choroid and sclera, embedded in OCT compound (Miles, Inc. Elkhart, IN), and frozen. The frozen tissue was sectioned with a cryostat at −20°C to a thickness of 10 μm and mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). For immunohistochemistry, the air-dried sections were preincubated with blocking reagent consisting of 4% goat serum, 1% BSA, and 0.4% Triton X-100 in PBS. The sections were incubated for 2 hours with primary antibody diluted 1:250 in PBS containing 1% goat serum, 1% bovine serum albumin, and 0.2% Triton X-100. The control sections were treated identically, except that the primary antibody was preincubated with 600 μg/ml peptide in excess to block the antibody binding sites. After washing, the sections were incubated with biotinylated goat anti-rabbit antibody. The bound antibodies were detected by incubation with a complex of avidin and biotinylated horseradish peroxidase from the Vectastain system (Vector Laboratories, Burlingame, CA) and assayed in a solution of the chromogen substrate, 0.04% 5-aminon-9-ethylcarbazole and 0.01% H₂O₂.

**RESULTS**

**Isolation of Preferentially Expressed RPE cDNA Clones**

A bovine RPE cDNA library in bacteriophage λg10 was screened for RPE-specific clones by differential hybridization to radiolabeled bovine RPE and kidney cDNA probes. Forty candidate cDNA clones were identified as having hybridized to the RPE probe but not to the kidney probe, and 10 of these were selected for further analysis by Northern blot hybridization. One of the selected cDNA clones, clone RPE6, hybridized to a 1.5 kb mRNA transcript that was seen in RNA from RPE, retina, and brain but not in RNA from other tissues (Fig. 1). A larger transcript, 3.4 kb in length, also hybridized prominently in RPE RNA. This larger mRNA was observed in retina RNA only after longer exposure, and it was not detected in brain RNA. The RPE6 cDNA clone contained a 1.3 kb insert that corresponded to an mRNA that is highly ex-
pressed in the RPE and that is expressed also in the retina and brain; however, it is barely detectable in the brain by Northern blot hybridization.

After rescreening the RPE cDNA library with the 1.3 kb cDNA as a probe, a clone with a longer cDNA insert and with an overlapping sequence (clone RPE12) was obtained. About 0.2% of the clones in the library contained the 1.6 kb a, -subunit probe was similar to that of the 1.3 kb cDNA. The inferred translational initiation context of a consensus translational start site. No signal peptide sequence was found at the predicted amino terminus. A desktop computer search of the nucleotide sequences within the GenBank database, release 74.0. The nucleotide sequence search was performed using the BLAST network service at the National Center for Biotechnology Information.

Sequence of a Novel G Protein-Coupled Receptor

The conceptual translation of the cDNA sequence yielded a long open reading frame of 291 amino acid residues with a cumulative molecular weight of 31,959 (Fig. 2). The putative initiation codon is the 5'-most ATG triplet of the cDNA sequence and is found in the context of a consensus translational start site. No signal peptide sequence was found at the predicted amino terminus. A desktop computer search of the
protein sequence database, Protein Identification Resource release 31.0, was conducted to identify homologous matches to the deduced amino acid sequence. A protein sequence similarity search was also performed using the BLAST network service. The searches resulted in best matches to a long list of known G protein-coupled receptors. The translated amino acid sequence showed significant homology to opsin, tachykinin, serotonin, and muscarinic receptors, all of which belong to the family of receptors having seven putative transmembrane domains. Within the family of heptahelical receptors, the query sequence was most highly conserved with members of the visual pigment lineage. It is about 25% identical and, taking into consideration the conservative amino acid substitutions, 42% similar to the sequence of bovine opsin. Its hydrophilicity plot (Fig. 3) and sequence alignment (Fig. 4) with bovine opsin do suggest the presence of seven hydrophobic segments in the novel amino acid sequence, each segment approximately 25 amino acids in length. As in bovine opsin, the cytoplasmic interfaces of the assigned transmembrane segments are consistently demarcated by or positioned close to a positively charged amino acid residue.

Glycosylation and intrachain disulfide bond formation are common posttranslational modifications of G protein-coupled receptors. A consensus sequence for potential glycosylation is located at Asn172 in the assumed second extracellular loop of the novel protein, but none is found in the amino terminal region (Fig. 4). Conserved disulfide bond formation involves cysteine residues located in the first and second extracellular loops of the heptahelical receptors. These potential sites for the common intrachain disulfide bond are conserved at Cys88 and Cys162 in the deduced amino acid sequence. The third cytoplasmic loop and carboxyl tail of the novel sequence contain several hydroxyl amino acids, some of which may be sites for regulatory phosphorylation. Because it contains homologous features and sequence motifs consistent with the structure of G protein-coupled receptors, the putative receptor may be referred to as an RPE-retinal G protein-coupled receptor, or denoted as RGR.

Expression of the rgr Gene and the Encoded Receptor Protein

To verify the expression of RGR in the RPE, in situ hybridization with RGR antisense RNA probes was performed using frozen sections of the tapetal region of normal bovine retina, where the RPE is amelanotic. The resulting autoradiogram revealed a highly specific pattern of RGR mRNA expression within the retina (Fig. 5). The antisense probe hybridized to the RPE cells, the inner nuclear layer, and specific cells in the ganglion cell layer, but it did not appear to hybridize to the photoreceptors. No signals were seen in the vascular endothelial cells. In the control experiment, hybridization with the sense RNA probe resulted in a low homogeneous background. The results demonstrate that RGR is expressed in the inner retina as well as in the RPE; however, the specific cell type in the inner retina is difficult to distinguish by in situ hybridization.

For investigation of the protein product of the rgr gene, antibodies were raised against a synthetic peptide corresponding to the carboxyl terminal amino acid sequence of RGR (CLSPQRREHSREQ). On Western blots, the affinity-purified antipeptide antibody recognized predominantly a 32-kD protein in bovine retina and RPE cell extracts, but not in extracts from other tissues (Fig. 6). The recognized protein agrees in size with the calculated molecular weight of the deduced RGR protein, and the distribution of the recognized protein is consistent with the pattern of RGR mRNA expression. In addition, the staining of this protein from the RPE was strong. Although the...

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933172/) Hydrophilicity profile of the amino acid sequence of bovine RGR. Hydrophilicity analysis of the translated RGR protein sequence revealed seven distinct hydrophobic regions. The positions of the hydrophobic segments I to VII are indicated, and, as shown in Figure 4, the hydrophobic segments closely align with the homologous transmembrane helices of bovine opsin. The analysis was performed according to the Kyte-Doolittle hydrophilicity scale using a window setting of 10 amino acids. Hydrophobicity increases with decreasing values.
FIGURE 4. Alignment of the amino acid sequence of bovine RGR with the sequences of bovine opsin and squid retinochrome. The amino acid sequence (single-letter code) of bovine RGR was aligned to that of bovine opsin and squid retinochrome. Transmembrane domains are shown for bovine opsin. Identical amino acid sequence matches to RGR are indicated by vertical lines. Horizontal dashes correspond to gaps in the sequences that allow optimal sequence alignment. The amino acid residues of each sequence are numbered on the right, beginning with the amino-terminal methionine. The sequence of retinochrome is shown in italics to specify a cross-species comparison. The lysine residue that serves as the retinaldehyde attachment site in rhodopsin is conserved in the putative seventh transmembrane domain of RGR. The amino and carboxyl terminal regions of the proteins are highly divergent. The sequence alignments were determined by the algorithm of Wilbur and Lipman using MacVector 3.5 software (IBI), and included minor adjustments to minimize gaps or insertions.

signal for the neural retinal extract was weak in comparison to the signal from the RPE extract, the relative signal intensities for neural retina and RPE may be skewed by the inclusion of a large amount of photoreceptors and other nonexpressing cells in extracts from the neural retina.

The affinity-purified antibody was used to localize RGR in bovine retina by immunohistochemistry. Frozen sections were prepared from bovine eyes that were light adapted and from regions of the retina in which the RPE monolayer was amelanotic. The retinal pattern of staining obtained with the RGR antibody was intrinsically specific, and the RPE monolayer was intensely positive (Fig. 7). In addition to the positive signal in the RPE, staining was observed in the region close to the inner limiting membrane and in cellular processes that extend from this region to the inner nuclear layer. This reproducible staining in the inner retina best represented localization to Müller cell processes and their basal end feet. The result is compatible with the in situ identification of hybridizing mRNA in the inner nuclear layer, where Müller cell nuclei reside. In the control, immunohistochemical staining was absent when the antibody was preincubated with excess peptide.

DISCUSSION

The retinal pigment epithelium consists of highly differentiated cells that have important roles in vision. In testing the hypothesis that specialized functions of the RPE involve cell-specific proteins, we have identified and cloned a novel heptahelical receptor from bovine RPE cells. This putative G protein-coupled receptor, referred to as RGR, is preferentially expressed in the RPE, the retina, and possibly the brain. It is not expressed in other tissues at the levels detected by RNA blot hybridization and protein immunoblot analyses. The putative RGR receptor appears to be derived from an abundant 1.5 kb mRNA. Although the 1.5 kb mRNA is the predominant species, a 3.4 kb transcript also cross-hybridizes with the RGR cDNA probe. The exact relationship between the two mRNA transcripts remains to be clarified. The restricted expression of
FIGURE 5. In situ hybridization to RGR mRNA in bovine RPE and retina. The photomicrographs show sections of normal bovine retina with amelanotic RPE that were hybridized to 35S-labeled (A) antisense and (B) sense bovine RGR RNA probes. The antisense probe hybridized to RPE cells, the inner nuclear layer, and specific cells in the ganglion cell layer. No clear signal was seen in the outer nuclear layer. In the control experiment, no signals were detected by hybridization with the sense RNA probe. The tissue sections were exposed for 12 days and stained with toluidine blue. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

RGR in the eye suggests that the function of this receptor is important in a visual process involving the RPE and specific cells in the neural retina.

Although we have not demonstrated biochemical pairing between RGR and G proteins, the deduced amino acid sequence of RGR clearly indicates that it is a member of the large family of receptors having seven transmembrane segments. Among the superfamily of heptahelial receptors, the putative RGR protein showed greatest overall homology to the visual pig-

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In contrast to the $\beta_2$-adrenergic receptor, the visual pigments are expressed at very high concentrations within differentiated organelles. Likewise, RGR is expressed at levels that appear to be much higher than the cellular levels of most other $G$ protein-coupled receptors. If the occurrence of RGR cDNA clones is comparable to relative protein abundance, then the amount of RGR protein may be within the order of 0.2% of total RPE cell protein. The intensity of staining on Western immunoblots also suggests that the RGR protein is present at high levels in the RPE. It is possible that the high concentration of RGR in the RPE indicates some importance for stoichiometry in its biochemical function.

The cellular retinaldehyde-binding protein (CRALBP) is another abundant protein in RPE and retina, with about 3 nmoles of CRALBP per bovine eye. Both CRALBP and cellular retinol-binding protein (CRBP) have been localized to the RPE and to Müller cells in the inner retina. These retinoid-binding proteins are thought to play a role in the visual cycle, and there is evidence that extracts from chicken neural retina, which contains Müller cells, can carry out the synthesis of 11-cis-retinoids from all-trans-retinol. At the light microscopic level, the immunohistochemical localization pattern for RGR protein coincides well with that of CRALBP and retinal CRBP, in that RGR is also localized in the RPE and Müller cells. Thus, at least three molecular markers in the retina intersect preferentially in RPE and Müller cells, indicating that there may be a common specialized function in the two cell types, involving one or more of the three proteins. RGR may be involved in the visual cycle or processing of retinoids in the RPE and Müller cells.

RGR also shows interesting parallels to squid retinochrome, a well-characterized photopigment. The retinochrome photoisomerase system has been described in the visual cells of cephalopods (squid and octopus) and arthropods (honeybee and blow fly). In these species, retinochrome binds to all-trans-retinal and, in the presence of light, reverses the chromophore to 11-cis-retinal. Like the photoisomerase, RGR in the RPE is localized away from, but close to, the primary site of phototransduction, and both proteins are abundantly expressed in the retina. In contrast to the visual pigments, RGR and squid photoisomerase do not contain putative glycosylation sites in their amino terminal domain, and both have relatively short amino and carboxyl terminal segments. It is possible that the amino terminal domain of RGR is longer than that indicated in Figure 2 if the actual translational start site is another ATG codon that lies farther upstream of the putative initiation codon. This possibility cannot be excluded by the present data; however, a much longer amino terminal domain would be unlikely because the size of the protein detected by
immunoblot analysis corresponds closely to that expected from the deduced amino acid sequence of RGR. The overall amino acid sequence of RGR is about 23% identical to that of the squid photoisomerase31 (Fig. 4). A region of RGR, from amino acid residue 139 to 189, is 45% identical and 76% similar in sequence to the region of squid photoisomerase, from residue 144 to 194. This sequence comparison was the highest-scoring segment pair resulting from a similarity search of protein sequence databases using the BLAST network service.

Although the function of RGR is completely unknown, the rgr gene appears to define a distant evolutionary branch of the visual pigment tree. If RGR evolved also as a retinal-binding protein, its identification in the RPE and Müller cells may have direct relevance for the molecular and genetic analysis of the vertebrate visual cycle. Alternatively, it is possible that RGR functions as a receptor for a novel ligand that acts selectively in the eye. Although the receptor function of RGR remains to be determined, the molecular cloning and characterization of the rgr gene may offer new insights into RPE function, differentiation, and disease.

Key Words
retinal pigment epithelium, Müller cell, visual pigments, retinochrome

Acknowledgments
The authors thank Dr. Steven Schreiber for assistance with the in situ hybridization and Dr. Suzanna Horvath for synthesis of the peptide antigens.

References


