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Structure and developmental expression of the mouse RGR opsin gene

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Purpose: The aim of this study is to isolate and characterize cDNA clones and the genes that encode mouse RPE retinal G protein-coupled receptor (RGR) and to analyze expression of the RGR gene in the developing mouse retina. The conserved amino acid sequences of RGR from various mammals can be compared to the amino acid sequence motif of G protein-coupled receptors.

Methods: Mouse RGR cDNA and gene clones were isolated from a retina cDNA library and 129SV genomic DNA library, respectively. The expression of RGR in the developing C57BL/6J mouse retina was analyzed by immunohistochemical staining with a polyclonal antipeptide antibody.

Results: The deduced amino acid sequence of mouse RGR is 78% and 81% identical to that of bovine and human RGR, respectively. The mouse RGR gene is split into seven exons and extends about 11 kb. Two predominant mRNA transcripts, 1.9 and 1.7 kb in length, and a third, relatively faint, 5.5-kb transcript were detected in mouse eye by hybridization to a RGR cDNA probe. Frozen sections of C57BL/6J mouse retina at various stages of development were incubated with a mouse RGR antipeptide antibody. RGR immunoreactivity was first seen at postnatal day 2 (P2) in centrally located RPE cells. From day P6 to P12, there was an increase in the number and intensity of immunoreactive RPE cells in the central and mid-peripheral regions of the retina, while the most peripheral RPE cells were still negative. By day P16, the length of the RPE monolayer was immunoreactive, and staining of the central RPE cells was markedly more intense than at younger ages.

Conclusions: Mouse and human RGR are highly conserved. A gradient of RGR expression in RPE extends from the central to the peripheral retina during development. In reference to the appearance of melanin-positive differentiated RPE cells, the induction of RGR expression is a relatively late event in the maturation of the retina.

The retinal pigment epithelium (RPE) is a monolayer of highly differentiated cells that are essential for the normal function of adjoining photoreceptors. Its diverse and unique roles in the visual process include the removal by phagocytosis of the discarded tips of photoreceptor outer segments [1] and the isomerization of all-*trans*to 11-*cis* retinoids for regeneration of visual pigments [2,3]. During development, the RPE and neuroretina originate in the optic cup from the outer and inner layers of neuroepithelial cells, respectively [4]. Melanin pigments are visible in the mouse RPE cell layer from embryonic day 12 (E12) [5]. Individual RPE-specific proteins begin to be expressed in the pigmented monolayer at various stages of cell maturation [6,7].

To perform its specialized functions, the RPE cell contains a panoply of specifically and preferentially expressed proteins. RPE-specific proteins include bestrophin [8], the RPE65 microsomal protein [9,10], 11-*cis*-retinol dehydrogenase [11,12], HMB-50 melanoma antigen [13], a monocarboxylate transporter protein (MCT3) [14], and RPE protective protein (RPP) [15]. The cellular retinaldehyde-binding protein (CRALBP) is expressed in the RPE, Müller cells, ciliary body, cornea, iris, and oligodendrocytes of the optic nerve and brain [16-18]. Peropsin, melanopsin and RPE retinal G protein-coupled receptor (RGR) are novel opsins that are found in RPE [19-21]. Notably, mutations in different RPE-specific or preferentially expressed genes are involved in inherited retinal degeneration [8,22-25].

The presence of multiple distinct opsins in RPE suggests that RPE cells are primary photoreceptive cells. The RGR opsin shares amino acid sequence similarity with visual pigments and retinochrome, a photoisomerase that catalyzes the conversion of all-*trans*- to 11-*cis*-retinal in squid photoreceptors [26]. RGR is an abundant integral membrane protein that is localized in the cytoplasm of RPE and Müller cells [27]. The protein has been purified from bovine RPE microsomal membranes in digitonin solution and has been shown to contain an endogenous chromophore. The shape of the absorption peaks and biochemical properties of the photopigment are consistent with those of a retinylidene Schiff base chromophore and reveal the existence of two pH-dependent species with absorption maxima at ~469 and ~370 nm [28].

On the basis of its unique subcellular localization and particular amino acid sequence, RGR offers a potential variation in the theme of opsin-related photopigments and G protein-coupled receptors. To study and compare the function and potential abnormalities of RGR in a mouse model system, we have obtained the deduced amino acid sequence of mouse RGR

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and characterized the structure and expression of the gene that encodes this nonvisual opsin.

METHODS

Isolation of cDNA and genomic DNA clones: Four mouse RGR cDNA clones were isolated from a λZAPII retina cDNA library after hybridization to a radiolabeled human RGR cDNA probe. One of these clones, MRGR7-5, contained a 1.5-kb cDNA insert, which was subcloned and sequenced completely on both strands. DNA sequencing was carried out using single and double strand phagemid DNA, sequence-specific primers, and Sequenase (U.S. Biochemical Corp., Cleveland, OH), according to the manufacturers' protocol. DNA clones containing the mouse RGR gene were isolated from a 129SV mouse genomic library in the λFIXII vector (Stratagene, Inc., La Jolla, CA). Five genomic clones (designated λmrgr9, λmrgr11, λmrgr12, λmrgr13 and λmrgr14) were identified by hybridization to the radiolabeled MRGR7-5 cDNA. A map of the mouse RGR gene was determined by complete and partial cleavage of *Not*I-digested genomic DNA using *Bam*HI, *Eco*RI, *Hind*III and *Sac*I restriction enzymes. The locations of the exons were mapped by oligonucleotide hybridization and amplification by the polymerase chain reaction.

RNA isolation and blot hybridization: Poly(A)+ RNAs from B6CBAF1/J mouse tissues were isolated using the Mini RiboSep mRNA Kit (Becton Dickinson Labware, Bedford, MA), according to the manufacturer's protocol. The RNA samples were electrophoresed in a 0.9% agarose gel containing 2.2 M formaldehyde and then transferred to a nitrocellulose filter. The filter was hybridized overnight at 42 °C in buffer containing 50% formamide, 5 x SSC, 50 mM NaH_2P0_4, pH 7.0, 2x Denhardt's solution, 0.1% SDS, 50 µg/ml denatured salmon sperm DNA, and a MRGR7-5 cDNA probe labeled with [α-^{32}P]dCTP by nick translation (10^6 count/min/ml). The final washing of the filter was performed in a solution containing 0.1 x SSC and 0.1% SDS at 50 °C for 30 min. Autoradiography was carried out by exposure to Kodak X-omat AR film at -80 °C using an intensifying screen.

Antibody production: A synthetic peptide that corresponds to the carboxyl terminal amino acid sequence (CLSPQKSKKDRTQ) of mouse RGR was conjugated to keyhole limpet hemocyanin (KLH) and used to generate rabbit antipeptide antisera. Antisera were obtained from Cocalico Biologicals (Reamstown, PA). The anti-mouse RGR antibody (mcDE5) was purified by means of an affinity chromatography column consisting of the synthetic peptide coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ).

Western blot analysis: For immunoblot analysis, B6CBAF1/J mouse tissues were homogenized with a Brinkmann polytron in a buffer containing 10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 250 mM sucrose, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 800 g, and membranes were then collected from the low-speed supernatant by centrifugation at 100,000 g for 30 min at 10 °C. The membrane pellets were suspended in homogenization buffer, and the proteins were electrophoresed in a 12% polyacrylamide-SDS gel, electroblotted onto nitrocellulose filter, and incubated with affinity-purified mcDE5 antibody. Specific binding of mcDE5 was detected by reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG, nitro blue tetrazolium, and 5-bromo 4-chloro 3-indolyl phosphate. Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry: Eyes from euthanized C57BL/6J mice at various stages of development were used for immunohistochemical study. The day of conception was designated as E0, and the day of birth as postnatal day 0 (P0). After the cornea was punctured to allow penetration of fixative, the eyes were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr at 4 °C. The eyecups were incubated overnight in 30% sucrose in phosphate-buffered saline (PBS) prior to embedding and freezing in OCT compound (Miles, Inc. Elkhart, IN). Tissue sections were cut with a cryostat to a thickness of 5-8 µm. After permeabilization with 0.2% Triton X-100 in PBS, the sections were treated for 30 min at room temperature with 3% bovine serum albumin (BSA) and 5% normal goat serum in PBS, followed by overnight incubation at 4 °C with affinity-purified mcDE5 antibody (diluted 1:100 in 1% BSA-PBS). The sections were washed and incubated at room temperature with biotinylated anti-rabbit IgG and then with FITC-conjugated streptavidin. The sections were covered with Vectashield (Vector Laboratories, Burlingame, CA), and immunostaining was visualized by epifluorescence microscopy using a Zeiss LSM-210 microscope. Color photomicrographs were obtained through a Sony UP-5000 dye sublimation printer.

All animals were treated, maintained, and euthanized in accordance with the ARVO resolutions on the use of animals in research and guidelines of the U.S. Public Health Service, as delineated in its Public Health Service Policy on Humane Care and Use of Laboratory Animals.

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MAATRALPAGLGELEVLAVGTVLLMEALSGISLNGLTIFSFCKTPDLRTPSNLLVLSLAL  60
--E-S---T-F---------M---V-----L---T----------E----CH--------
--ESGT--T-F-------------V-----L---I---L------E-----H--------

ADTGISLNALVAAVSSLLRRWPHGSEGCQVHGFQGFATALASICGSAAVAWGRYHHYCTR  120
--S----------T--------Y--D---A------V-------S---I-----------
--S----------T--------Y------A------V-------S-----------F---

RQLAWDTAIPLVLFVWMSSAFWASLPLMGWGHYDYEPVGTCCTLDYSRGDRNFISFLFTM  180
S----NS-VS------L------A---L---------L---------K-----T------
SR-D-N--VS--F---L------A---L---------L---------------T------

AFFNFLVPLFITHTSYRFMEQKFSRSGHLPVNTTLPGRMLLLGWGPYALLYLYAAIADVS  240
S----AM-----I---SL----LGK----Q------A-T---------I-----V----T
------L-----VV---L----LGKTSRP----V--A-T---------------T---AT

FISPKLQMVPALIAKTMPTINAINYALHREMVCRGTWQCLSPQKSKKDRTQ* mouse    291
S--------------MV----------GN------I--------RE----K* human
S--------------AV--V--M----GS---H--I-------RREHS-E-* bovine
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Figure 1. Amino acid sequence of mouse RGR aligned with that of human and bovine RGR. For comparison, only amino acid (one-letter code) sequence differences in the human and bovine proteins are shown, and identities to mouse RGR are indicated by dashes. The sequence of the carboxyl terminal peptide used to generate the mcDE5 antibody is shown in blue, and numbering of the amino acid sequence is on the right. A conserved lysine residue, which is homologous to the retinal attachment site of visual pigments, is shown in red. The nucleotide sequence of the mouse RGR cDNA is deposited under GenBank accession number AF076930.

RESULTS

Characterization of mouse RGR cDNA and genomic DNA clones: The MRGR7-5 cDNA clone was isolated from a mouse retina cDNA library and was shown to contain the entire protein-coding region of RGR. The sequence of MRGR7-5 cDNA is 1493 nucleotides in length (deposited under GenBank accession number AF076930). Translation of the cDNA sequence from its 5'-most ATG codon to the in-frame stop codon yields an open reading frame of 291 amino acids with a calculated molecular weight of 32,124 (Figure 1). The deduced amino acid sequence of mouse RGR is 81% and 78% identical to that of human and bovine RGR, respectively. Lys255, homologous with the retinaldehyde attachment site in visual pigments, is conserved in the seventh transmembrane domain of RGR from each species.

Two overlapping genomic DNA clones, λmrgr9 and λmrgr11, were used to obtain a restriction map of the entire 129SV mouse RGR gene (Figure 2). The gene is split into seven exons and spans approximately 11 kb. The exon-intron junctions in the mouse RGR gene correspond to those in the human gene.

Tissue-specific expression of RGR: The expression of RGR mRNA in mouse tissues was analyzed by Northern blot hybridization using the MRGR7-5 cDNA as probe. In mouse eye, three mRNA transcripts were detected (Figure 3). The two major transcripts were 1.9 and 1.7 kb in length. A faint third transcript, 5.5 kb in length, was also found. No hybridizing mRNA transcripts from liver, kidney or brain were detectable in this assay.

Since previously generated antibodies directed against bovine and human RGR did not cross react with mouse RGR, a synthetic peptide that corresponds to the carboxyl terminal amino acid sequence of mouse RGR was used to produce the antibody, designated mcDE5. The affinity-purified mcDE5 antibody reacted with a single 31-kDa protein on immunoblots of membrane proteins from mouse eyes, but did not detect any protein from liver, kidney or brain (Figure 4). The 31-kDa protein was similar in size to bovine RGR from the RPE and retina, and its observed tissue-specific expression pattern was consistent with that of RGR mRNA.

Expression of RGR in the developing mouse retina: The mcDE5 antibody was used to determine the localization of RGR in mouse retina by immunohistochemical staining. As expected, specific immunoreactivity was observed reproducibly in adult mouse RPE cells (results not shown). The signal was intense and continuous throughout the RPE monolayer from the central to the peripheral region. No staining was seen in the photoreceptors or other neurons of the retina. In contrast to the immunohistochemical staining of RGR in human and bovine retinas, the staining of RGR in mouse Müller cells was barely detectable.

To investigate the expression pattern of RGR during mouse retina development, a series of eyecup sections from mice at E10 to P16 was incubated with the mcDE5 antibody. No RGR immunoreactivity was seen in the mouse retinas at E10, E18, or on the day of birth. At developmental stage P2, immunofluorescent signals were confined to a few RPE cells in the central retina, and no staining was detectable in the peripheral retina (Figure 5). From P6 to P12, immunostaining of the RPE cells in the central and mid-peripheral regions of the retina increased in strength and contiguity, while RPE cells in the far-peripheral region were negative or showed weaker staining. After P16, RGR immunoreactivity was detectable throughout the RPE monolayer. Overall, the induction of RGR expression follows a central to peripheral gradient, and it occurs during a ~2 week period following the time of birth.

DISCUSSION

Recent studies have revealed novel opsin-related photopigments in the RPE and other non-photoreceptor cells [19-21,29,30]. The RGR gene is derived from a distant evolutionary branch of the vertebrate opsin-visual pigment family and has a distinct organization of exons and introns. To pursue a genetic approach to understanding the biological function of RGR, we isolated and investigated the RGR gene in mice.

Comparison of mammalian RGR: The amino acid sequences of mouse, human and bovine RGR are 78-86% identical between the three proteins. Divergent substitutions between the proteins are relatively numerous in the amino and

Figure 2. Restriction map and structural arrangement of the mouse RGR gene. A restriction map of *Bam*HI, *Eco*RI, and *Sac*I sites in the 129SV mouse RGR gene was determined from overlapping genomic DNA clones, λmrgr9 and λmrgr11. The positions of exons 1-7 are represented by the solid boxes.

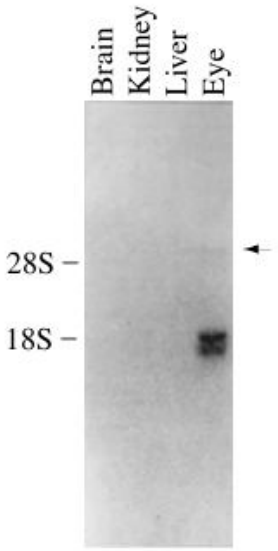

Figure 3. Northern blot hybridization. Poly(A)+ RNAs from mouse eye (0.6 µg) and other tissues (10 µg) were hybridized to a ^{32}P-labeled probe, derived from the mouse cDNA clone MRGR7-5. An arrow points to the 5.5-kb mRNA transcript in mouse eye.

carboxyl terminal domains. Despite the amino acid sequence differences, one property that is conserved at the carboxyl terminus of RGR is the presence of many highly charged amino acid residues, which suggests that electrostatic interactions at the carboxyl terminus may be involved in the function or regulation of RGR. Lys255 and His91 are conserved in RGR from the three species, and their positions correspond to those of two critical amino acids in visual pigments. Lys255 is homologous with the conserved lysine residue that serves as the retinaldehyde attachment site in visual pigments. His91 takes the position of the retinylidene Schiff base counterion (Glu113 in bovine rhodopsin) [31-33]; however, there is no evidence yet that His91 is located close to the retinylidene Schiff base in RGR. In sharp contrast to vertebrate visual pigments, the observed p*K*a of the protonated Schiff base in bovine RGR is ~6.5, and the absorption spectrum of the isolated protein contains pH-sensitive absorption maxima at ~469 and ~370 nm [28].

RGR belongs to the family of G protein-coupled receptors, and amino acid residues that conform to a distinctive sequence pattern in G protein-coupled receptors [34] are conserved in mouse, human and bovine RGR. The amino acid sequence of RGR contains the Arg113-Tyr114 (RY) sequence, which corresponds to part of a highly conserved sequence motif in G protein-coupled receptors. The ERY (or DRY) sequence motif is found in nearly all G protein-coupled receptors, but it is replaced by a unique GRY sequence in RGR. The arginine of the ERY sequence motif in rhodopsin is required for rhodopsin to activate transducin, whereas the neighboring glutamate residue appears to inhibit activation [35,36]. Replacement of glutamate with glutamine in the E134Q mutant of rhodopsin does not abolish the ability of the visual pigment to activate transducin. Instead, the ability of the mutant to activate transducin is enhanced [37,38].

In addition to Lys255, a few other polar amino acids are conserved within the transmembrane domains of mammalian RGR. The first, second and sixth transmembrane segments contain Glu26, Asp62 and Arg218, respectively. The presence of these conserved hydrophilic amino acids in a transmembrane domain suggests a high degree of selection for the charged residues. Asp62 in helix 2 is highly conserved among the G protein-coupled receptors and is thought to be involved in receptor activation and the stimulation of interaction with G proteins [39]. The hydrophilic Glu26 and Arg218 residues in the transmembrane segments are unique in RGR and are not part of the conserved amino acid sequence motif of G protein-coupled receptors [34].

Interestingly, there are a few sequence similarities between RGR and the vertebrate visual pigments in the cytoplasmic loops. Both RGR and visual pigments contain the highly conserved amino acid sequence motifs, Lys-Arg-Xxx-Pro and Gln-Lys-Xxx-Xxx-(Lys/Arg), in the first and third cytoplasmic loops, respectively. The Lys205 residue in bovine RGR appears to correspond to bovine rhodopsin Lys248, the mutation of which significantly reduces transducin activation [38].

Mouse RGR gene: The mouse and human RGR genes are well conserved, and the structure of each contains seven exons. In the mouse eye, the RGR gene is transcribed into two major and one minor mRNAs. The multiple mRNAs may result from alternative processing of mouse RGR pre-mRNA. The existence of a closely related novel gene cannot be excluded by the present data; however, no such RGR-related

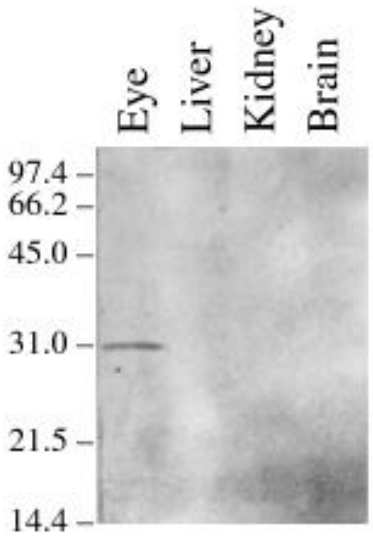

Figure 4. Immunoreactivity of affinity-purified anti-mouse RGR antibody. Membrane proteins (100 µg) of each tissue were immunoblotted and incubated with the mcDE5 antipeptide antibody, which recognizes a single ~31-kDa protein in the eye. The molecular masses of protein standards are indicated in kDa at the left.

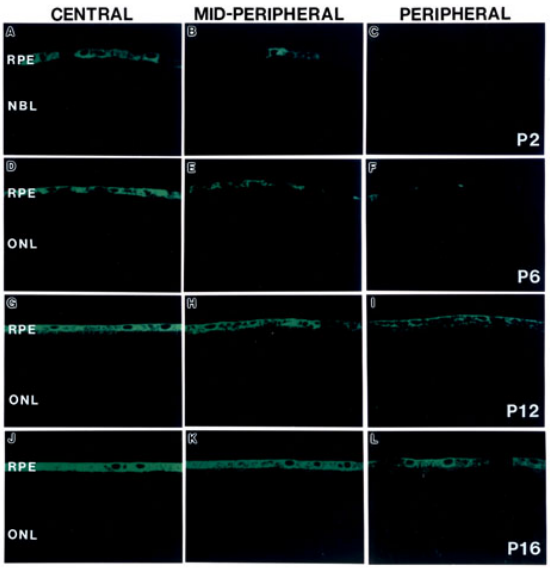

Figure 5. Expression of RGR in the developing mouse retina. Immunofluorescence was observed initially in RPE cells in the central retina at developmental stage P2 and increased thereafter from the central to the peripheral retina. The RPE cells during the embryonic period show no immunoreactivity (results not shown). The series of images encompasses the choroid, RPE and the outer portion of the neuroretina at P2 (A-C), P6 (D-F), P12 (G-I) and P16 (J-L). The neuroblastic layer (NBL) and the outer nuclear layer (ONL) are labeled.

gene has been identified by probe hybridization to several cDNA and genomic DNA libraries.

During development of the mouse retina, the RGR protein is expressed initially at P2 and is confined to the central RPE cells. The pattern of RGR expression then follows a central to peripheral gradient and parallels closely the temporal and spatial maturation of rod photoreceptors. Thus, in comparison to early RPE cell genesis [40], the expression of RGR protein and the maturation of rod photoreceptors are both late events in retinal development with similar periods of duration. Another RPE-specific protein, RPE65, is also first expressed late in retinal development. RPE65 is detected initially at P4 in the centrally located RPE cells of the neonatal rat eye [7]. In contrast, CRALBP and tyrosinase proteins appear much earlier in the developing RPE and are first detected in prenatal rat RPE cells at E13 [6,41]. It is unknown whether common regulatory signals are involved in the maturation of photoreceptors and the regulation of late RPE-specific gene expression. The mouse RGR gene provides a model to investigate the developmental control mechanisms in RPE cells at the level of transcription or translation.

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