Molecular Vision 2007; 13:1203-14 <http://www.molvis.org/molvis/v13/a131/>
Received 5 October 2006 | Accepted 25 June 2007 | Published 19 July 2007

Deposition of exon-skipping splice isoform of human retinal G protein-coupled receptor from retinal pigment epithelium into Bruch's membrane

Meng-Yin Lin,[1] Harold Kochounian,[2] Roger E. Moore,[3] Terry D. Lee,[3] Narsing Rao,[1,4] Henry K. W. Fong,[1,2,4]

(The first two authors contributed equally to this publication)

[1]Doheny Eye Institute, Los Angeles; [2]Department of Molecular Microbiology and Immunology, Keck School of Medicine of the University of Southern California, Los Angeles; [3]Division of Immunology, Beckman Research Institute of the City of Hope, Duarte; [4]Department of Ophthalmology, Keck School of Medicine of the University of Southern California, Los Angeles

Purpose: Human retina and retinal pigment epithelium (RPE) express a relatively abundant mRNA that encodes an extraneous splice isoform of the RPE retinal G protein-coupled receptor (RGR) opsin. In this study, we investigate this exon-skipping RGR splice isoform (RGR-d) in separated neural retina and RPE cells of human donors of various ages.
Methods: We used mass spectrometry, sensitive western blot assay, immunohistochemical localization and real-time RT-PCR to analyze RGR-d.
Results: Western blot assay detected the RGR-d protein in the neural retina of all donors analyzed. Mass spectrometric analysis of the immunoreactive proteins independently confirmed the presence of RGR-d. In contrast, RGR-d protein in the RPE of most donors was barely detectable by western blot assay, even though expression of RGR-d mRNA was confirmed by amplification of RGR-d transcripts in both the RPE and neural retina. Quantitative real-time RT-PCR assays showed that RGR-d/RGR mRNA transcript ratios were about 0.17 and about 0.33 in the RPE and neural retina, respectively. Immunohistochemical localization studies revealed that the RGR-d epitope was present near the basal boundary of RPE cells and primarily in the extracellular areas of Bruch's membrane, adjacent choriocapillaris, and intercapillary region of both young and older donors. Positive immunostaining was seen in the drusen of older individuals.
Conclusions: The RGR-d protein is a common mutant form of human RGR that can be identified in donor eyes by mass spectrometry. These results indicate that after RGR-d is synthesized, the RGR-d epitope is released at the basal surface of the RPE and deposited into Bruch's membrane in human eyes throughout adult life.

Alternative splicing of pre-mRNA is a common phenomenon that leads to expansion of the proteome and plays a major role in regulating gene function. It has been established that pre-mRNA from as many as half of all human genes undergo alternative splicing [1-7]. An important question is whether the many splice variants represent functional alternative splicing. In some cases, there is no evident function for the splice isoform, or there is no evidence that the protein is actually produced. Unproductive mRNA transcripts, which contain premature termination codons, are widely expressed and become targets of nonsense-mediated mRNA decay [8,9]. Rare splicing errors may occur by chance through the influence of *cis*- and *trans*-acting splicing factors. Other seemingly nonfunctional mRNAs are of biological origin and are legitimate [10-12].

We have found a relatively abundant, presumably nonfunctional mRNA that is expressed in human, but not in mouse or bovine, retina and retinal pigment epithelium (RPE) [13]. This mRNA encodes a splice isoform of the human RPE retinal G protein-coupled receptor (RGR) opsin, which is a membrane-bound protein preferentially expressed in the RPE and Müller cells of the retina [14]. RGR opsin belongs to the family of G protein-coupled receptors, being most homologous to peropsin, an opsin localized to the apical microvilli of RPE [15], and retinochrome, a photoisomerase that catalyzes the conversion of all-*trans*- to 11-*cis*-retinal in squid photoreceptors [16]. RGR carries all-*trans*-retinal as its endogenous chromophore and converts the all-*trans* isomer stereospecifically to 11-*cis*-retinal upon irradiation [17]. Studies of RGR knockout mice indicate that RGR is necessary to maintain a normal rate of 11-*cis*-retinal synthesis both in light and in darkness after irradiation [18-20]. Mutations in the human *RGR* gene are associated with retinitis pigmentosa [21].

There are two known splice isoforms of human RGR in the retina. One variant (RGR-i) results from the alternative use of an internal acceptor splice site in the second intron [22]. RGR-i contains an insertion of four amino acids in the connecting loop between the second and third transmembrane domains. A second isoform (RGR-d) results from an inframe deletion of exon 6 sequences [13]. We have found the RGR-d mRNA in all postmortem donor retinas examined thus far. A corresponding RGR-d mRNA transcript is not conserved in the bovine or mouse retina. We have shown by immunodetection that the RGR-d protein exists in the retinas

Correspondence to: Henry K. W. Fong, Doheny Eye Institute, 1355 San Pablo Street, Los Angeles, CA 90033; Phone: (323) 442-6675; FAX: (323) 442-6688; email: hfong@usc.edu

of a large proportion of individual donors, including patients with age-related macular degeneration [23].

The RGR-d isoform is intriguing because it appears to be a nonfunctional, mutant form of RGR that is common in humans. RGR-d may affect the function of RGR insofar as, the more RGR pre-mRNA is spliced to RGR-d, the less normal RGR will be expressed. Since other mutants of RGR are associated with inherited retinal degeneration [21], there is a possibility that the RGR-d protein is pathogenic under certain conditions, or that it contributes to the progressive derangement of RPE cells that is prevalent in older humans [24,25].

In this paper, we investigate RGR-d in the neural retina and RPE cells of individuals at various ages. We analyze the splice isoform by mass spectrometry, sensitive western blot assay, immunohistochemical localization, and amplification of cDNA. Our results show that the RGR-d protein is detectable in the retina of each donor and that an RGR-d epitope is present near the RPE in the extracellular region of Bruch's membrane and adjacent intercapillary region.

METHODS

Antibody production: Affinity-purified RGR and RGR-d antibodies were produced as described previously [13,23]. The HRGR-DE7 antipeptide antibody was generated against the carboxyl terminal amino acid sequence of human RGR (CLSPQKREKDRTK). The DE17 and DE21 antipeptide antibodies were generated against the unique junction sequence of human RGR-d (GKSGHLQVPALIAK) at the conjoined splice point of exons 5 and 7. The peptides to be used as antigens were synthesized (by Suzanna Horvath, California Institute of Technology) according to amino acid sequences deduced from human RGR and RGR-d cDNAs. The carboxyl terminal and RGR-d peptides were conjugated with succinimidyl 4-(N-maleimido methyl) cyclohexane-1-carboxylate to keyhole limpet hemocyanin for immunization. Rabbit antisera were produced by Cocalico Biologicals (Reamstown, Pennsylvania). An emulsion of 100 μg immunogen in an equal volume of complete Freund's adjuvant was injected subcutaneously, and after 14 days, the rabbits were boosted with 50 μg immunogen in an equal volume of incomplete Freund's adjuvant. The antibodies were affinity-purified from 9-24 ml of antiserum by specific binding to the immobilized peptide attached to CNBr-activated Sepharose (Pharmacia Biotech), or Affi-Gel 10 resin (Bio-Rad, Hercules, California). The synthetic peptides (7.2-14.5 mg) were conjugated to the resins according to the manufacturer's protocol. Aliquots of affinity-purified antibodies were stored at -80 °C.

Preparation of retina and retinal pigment epithelium membrane protein: Postmortem eyes (eight donors, 18-77 years of age; Table 1) were obtained from the Doheny Eye and Tissue Transplant Bank (Los Angeles, California) and the National Disease Research Interchange (Philadelphia, Pennsylvania). After excision of the cornea, lens and vitreous, the neural retina was carefully removed. The eyecup was washed with ice-cold phosphate-buffered saline (PBS) to remove any remaining retina. The RPE cells were then collected in PBS by gently scraping the cell monolayer with a spatula. The retina and RPE cells were frozen in liquid nitrogen and stored at -80 °C. Each sample was later thawed on ice and homogenized in ice-cold buffer containing 67 mM sodium phosphate, pH 6.7, and 250 mM sucrose with the use of a Dounce glass homogenizer or mechanical polytron. After the homogenate was centrifuged at about 700 g for 10 min at 4 °C, the pellet was resuspended in buffer and homogenized again. The combined supernatant was then centrifuged at >100,000xg for 1 h at 4 °C. The final pellet was resuspended in the homogenization buffer and stored at -80 °C.

Western blot analysis: Proteins were electrophoresed in a 12% polyacrylamide-0.1% sodium dodecyl sulfate gel (SDS-PAGE) and then transferred to Immobilon-P membranes (Millipore Corp., Bedford, Massachusetts). The blots were initially incubated with affinity-purified primary antibody at ambient temperature, and then with a secondary antibody that was conjugated to horseradish peroxidase. Immunoreactive antigens were detected by chemiluminescence using the horseradish peroxidase -based ECL (Amersham, Arlington Heights, Illinois) or SuperSignal West Femto (Pierce Biotechnology, Rockford, Illinois) substrate systems. Chemiluminescence was detected by exposure to BioMax XAR film. The density of protein bands was determined with the Scion Image 1.63 software (Scion Corp., Frederick, Maryland). ECL Immobilon-P blots were reprobed by stripping, bound antibodies from the blot and washing them at 50 °C for 30 min in buffer containing 62 mM Tris-HCl, (pH 6.7), 100 mM β-mercaptoethanol, and 2% SDS.

A recombinant human RGR-d protein was produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, California), as described previously [23]. A 1.1-kb cDNA encoding human RGR-d was excised from the clone pcDNA3-hRGR-d by digestion with EcoR1 and inserted into the pFastBac donor plasmid. DH10Bac *E. coli* competent cells were transformed with the pFastBac/RGR-d plasmid to generate an expression bacmid. *Spodoptera frugiperda* (*Sf9*) cells were cultured in serum-free medium, Sf-900 II SFM, at 27 °C in a non-humidified, ambient air incubator. The cells were transfected with the baculovirus RGR-d expression bacmid to produce stocks of recombinant RGR-

Table 1. Information on postmortem donor eyes

Donor	Age/Gender	Known ocular history[1]	Postmortem interval[2]
CV-008	73/F	none	27
CA-050	73/F	none	75
CA-066	59/M	RD	42
Y-005	20/F	none	22
Y-116	18/M	none	37
Y-214	21/M	none	45
CA-0022	77/F	none	nd
960105	69/M	MD (eyebank)	34

Known ocular history is from information provided by the eye banks. The postmortem interval time is the approximate hours from time of death to time of tissue processing or freezing at -80 °C. RD represents retinal degeneration; MD represents macular degeneration; nd represents not determined.

d baculovirus. The viral titer was determined, and the viral stock was amplified to $1x10^8$ pfu/ml. The optimal infection conditions were tested at various multiplicity of infection. Whole cell extracts from RGR-d baculovirus- transduced cells and untreated control *Sf9*cells were prepared by lysing the infected cells in gel loading buffer containing 62.5 mM Tris-HCl, (pH 8.0), and 2% SDS. A western blot using the HRGR-DE7 antibody was performed to assay the expression of RGR-d.

Mass spectrometric analysis of retinal G protein-coupled receptor splice isoform: Proteins from the retina and RPE of individual donors were electrophoresed in a 12% SDS-polyacrylamide gel and stained with Coomassie blue. The protein bands of interest were excised from the gel and treated with trypsin for in-gel digestion [26]. A portion of the digested peptide mixture was analyzed by liquid chromatography-mass spectrometry (LC/MS/MS) using an Eksigent NanoLC-2D HPLC system (Eksigent Technologies, Dublin, California) interfaced directly to an LTQ-FT mass spectrometer (ThermoElectron, San Jose, California). The mass spectrometer was set to collect fragment ion spectra of the singly and doubly protonated ions of the splice-site peptide in alternating scans. The identity of the analyzed peptides was confirmed by comparing their spectra to reference spectra obtained from a synthetic form of the splice-site peptide.

Analysis of human retinal G protein-coupled receptor mRNA by amplification: Total RNA was isolated from human donor neural retina and RPE cells using RNA-Bee RNA isolation reagents from Tel-Test, Inc. (Friendswood, Texas), according to the manufacturer's instructions. Each tissue was added to 0.5 ml RNA-Bee solution and sheared by passing through a 21-22 gauge needle. After further addition of 0.5 ml RNA-Bee, the sample was homogenized in a 7 ml glass dounce homogenizer. The solution phases were separated, and the RNA was precipitated.

Human RGR mRNA transcripts were analyzed by reverse transcription and amplification by polymerase chain reaction (RT-PCR) using the ThermoScript RT-PCR system from Invitrogen. At least 20 ng of total RNA from the RPE or neural retina were used in the analysis. The RNA and primer were denatured at 65 °C for 5 min. The reverse transcriptase reaction was performed with 50 pmol of oligo(dT), at least 20 ng RNA, and 1 ml of ThermoScript RT (15 U/ml) in a 20 ml volume reaction for 1 h at 55 °C. RNaseH (2 U/ml) was added to each reaction at 37 °C for 20 min.

A region of the RGR cDNA was amplified using primers S200 (CCC TAC GGC TCG GAC GGC TGC) and A860 (CTT CTG CGG TGA GAG GCA). Amplification with the S200 and A860 primers yielded expected fragments of 609 and 495 nucleotides in length, which correspond to the intact RGR and truncated RGR-d mRNA, respectively. The PCR was performed with 10 pmol of each primer, 2 µl of the first-strand cDNA solution (or RNA solution without reverse transcriptase, as a negative control), and 2.5 units of platinum *Taq* polymerase for 40 cycles of 45 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C. The fragments were electrophoresed and visualized with ethidium bromide.

Real-time polymerase chain reaction analysis of retinal G protein-coupled receptor and retinal G protein-coupled receptor splice isoform: The Roche LightCycler 480 real-time PCR system (Roche Applied Science, Indianapolis, Indiana) was used to analyze human RGR and RGR-d mRNA. Hydrolysis probes were from the Universal ProbeLibrary (Roche

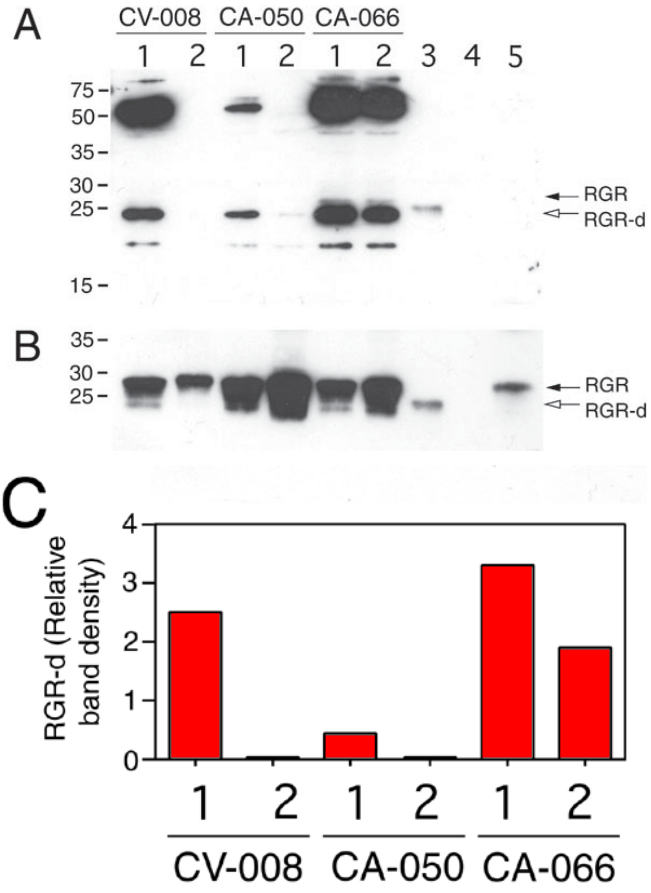

Figure 1. Western immunoblot assay of retinal G protein-coupled receptor splice isoform and retinal G protein-coupled receptor in human retina and retinal pigment epithelium. **A**: The expression of retinal G protein-coupled receptor splice isoform (RGR-d) in the neural retina (lane 1), and retinal pigment epithelium (RPE; lane 2) from donors CV-008 (73/F), CA-050 (73/F), and CA-066 (59/M) was analyzed on western blots using the DE17 antibody. **B**: The blot shown in **A** was stripped of bound antibodies and incubated subsequently with the HRGR-DE7 antibody. **C**: Relative level of RGR-d in reference to the amount of RGR. Initially, samples were loaded onto the gel so that RGR would be about equal intensity in each lane. The relative density of RGR-d bands (**A**) was then normalized to the band density of RGR (**B**). Controls included RGR-d baculovirus-transduced *Spodoptera frugiperda* (*Sf9*) whole cell extract (lane 3), and an equal amount of control *Sf9* whole cell extract (lane 4). Human RGR protein (lane 5) was solubilized in 1% Triton X-100/PBS and isolated from RPE cells of a single donor (CA-014, 84-year-old female) by an immunoaffinity procedure [23]. Monomer RGR-d (open arrows) and full-length RGR (solid arrows) were detected by chemiluminescence using (**A**) SuperSignal West Femto and (**B**) ECL™ substrate systems. Donor CA-066 was diagnosed with retinal degeneration and insulin-dependent diabetes mellitus.

Applied Science). Synthetic oligonucleotides were obtained from Invitrogen (Carlsbad, California). For this analysis, the following primer-probe sets were used: RGR upstream (exon 5), 5'-TTC CTA CAG TCT CAT GGA GCA G-3'; RGR downstream (exon 6), 5'-TGC GTA TAG ATA CAG GAT GGC ATA-3'; RGR hydrolysis probe, 5'-TCT CCA GG-3'; RGR-d upstream (exons 5 and 7), 5'-AGT GGC CAT CTC CAG GTG C-3'; RGR-d downstream (exon 7), 5'-ATT CCC CTG CAG ACC ATC T-3'; RGR-d hydrolysis probe, 5'-CTG GGC AA-3'. We analyzed the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as a reference. The primer-probe set that we used for GAPDH is as follows: GAPDH upstream, 5'-AGC CAC ATC GCT CAG ACA-3'; GAPDH downstream, 5'-GCC CAA TAC GAC CAA ATC C-3'; GAPDH hydrolysis probe. 5'CTG CTG GG-3'.

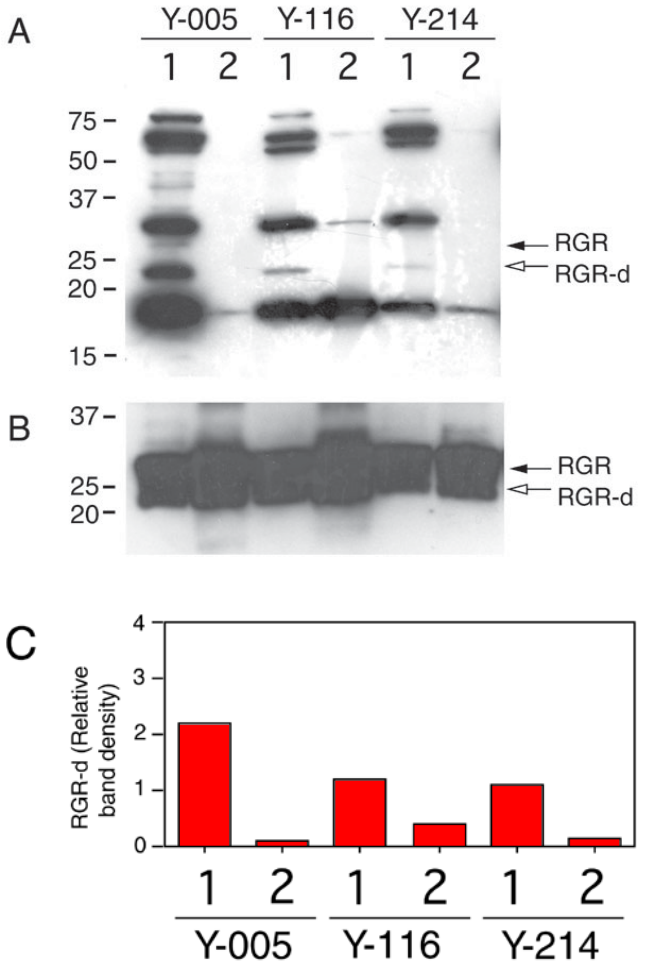

Figure 2. Western immunoblot assay of retinal G protein-coupled receptor splice isoform and retinal G protein-coupled receptor in the retina and retinal pigment epithelium of young donors. A single blot of the neural retina (lane 1) and retinal pigment epithelium (RPE; lane 2) membrane proteins from donors Y-005 (20/F), Y-116, (18/M), and Y-214 (21/M) was prepared so that retinal G protein-coupled receptor (RGR) would be about equal intensity in each lane. The blot was (**A**) incubated with the DE17 antibody, strip-washed, and then (**B**) incubated with the HRGR-DE7 antibody. **C**: The relative density of retinal G protein-coupled receptor splice isoform (RGR-d) bands was normalized to the band density of RGR. Monomer RGR-d (open arrows) and full-length RGR (solid arrows) were detected by chemiluminescence using (**A**) SuperSignal West Femto and (**B**) ECL™ substrate systems.

Samples of total RNA from the retina and RPE were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). For negative controls, RNA was incubated without reverse transcriptase. Real-time PCR assays were performed in triplicate. A portion of each first-strand cDNA reaction was mixed with 100 nM hydrolysis probe, 200 nM each of the forward and reverse PCR primers, and LightCycler® 480 Probes Master (Roche) solution in a final volume of 20 µl. The LightCycler 480 was programmed for 1 cycle of pre-incubation at 95 °C for 5 min, 45 cycles of heating at 95 °C for 10 s and amplification at 61 °C for 25 s, and 1 cycle of cooling at 40 °C for 10 s. Plasmids containing human RGR and RGR-d cDNAs were used to determine standard curves.

Immunohistochemistry: Tissue bank eyes were either processed without fixation or they were fixed with 4% paraformaldehyde in PBS for 4-6 h at 4 °C. Fixed tissues were infiltrated overnight with 30% sucrose in PBS. The retinas were dissected from the sclera, embedded in Tissue-Tek O.C.T. Compound (Sakura, Torrance, California) and frozen. The frozen tissues were sectioned with a cryostat at -20 °C to a thickness of 5-8 µm, mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania), air-dried, and treated with cold acetone for 5 min. Retina sections were permeabilized by incubation with 0.2% Triton X-100 in PBS or treated with blocking reagent, consisting of 3% bovine serum albumin, 5% normal goat serum and 0.2% dodecylmaltoside in PBS. After blocking, the sections were incubated with affinity-purified primary antibodies at ambient temperature or 37 °C. Immunohistochemical staining was performed with a peroxidase-based enzyme detection system (Vectastain ABC or Impress, Vector Laboratories, Burlingame, California) using the Vector VIP substrate, according to the manufacturer's instructions. Control slides were treated in the same manner, except that the primary antibody was either omitted from the binding buffer, or blocked by pre-adsorption with 100 µM RGR-d peptide in PBS for 30 min. The sections were mounted in aqueous Crystal/Mount medium, or they were dehydrated sequentially with 95% and 100% ethanol, cleared with xylene, and covered with VectaMount (Vector Laboratories). Images were photographed using a Leica DM LB2 microscope system with Spot digital camera and Advanced version 4.0.8 (Diagnostic Instruments, Inc., Sterling Heights, Michigan).

RESULTS

Relative level of retinal G protein-coupled receptor splice isoform protein in human retina and retinal pigment epithelium: Retina and RPE membrane proteins from individual donors (ages 59-73) were analyzed on a western blot by incubation with affinity-purified HRGR-DE7 antibody and DE17, an antibody directed preferentially against RGR-d (Figure 1). DE17 reacted specifically with recombinant RGR-d and several prominent bands (about 24, about 55, and about 18 kDa)

in the retina of each donor (Figure 1A). In the RPE of donors CV-008 and CA-050, DE17 reacted only faintly, if at all, to these proteins. In donor CA-066, the bands from the RPE were about half as intense as the bands from the retina. HRGR-DE7 bound both recombinant RGR-d and normal RGR (Figure 1B). The protein gel was loaded so RGR would be about equal intensity in the retina and RPE of each donor. The relative intensity of RGR-d bands to that of full-length RGR varied significantly, most especially in the RPE, for which the intensity ratio of RGR-d to RGR bands was extremely low for donors CV-008 and CA-050 (Figure 1C). The results also indicate that the DE17 antibody did not cross-react with intact RGR and that retina proteins did not contaminate the RPE sample to a large degree.

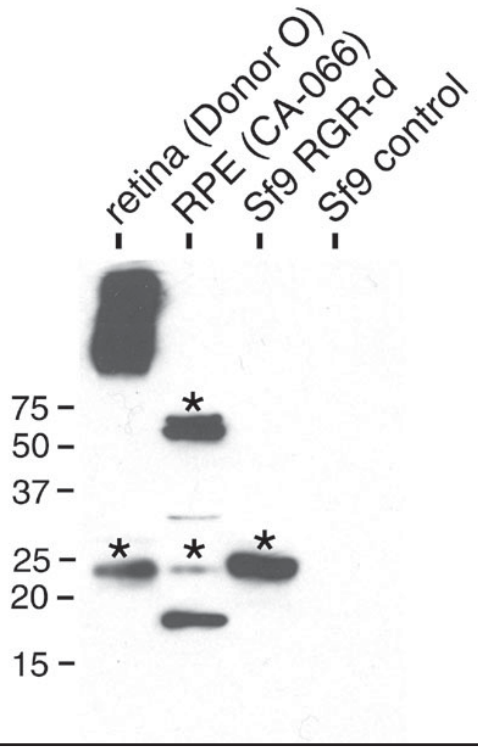

Figure 4. Western immunoblot assay of retinal G protein-coupled receptor splice isoform in the neural retina of donor O, retinal pigment epithelium of donor CA-066, and baculovirus-transduced *Spodoptera frugiperda* whole cell extract. Untreated *Spodoptera frugiperda* (*Sf9*) whole cell extract served as a negative control. The blot was probed with the DE17 antibody. The asterisks indicate protein bands that were cut from a parallel gel for liquid chromatography-mass spectrometry (LC/MS/MS) analysis. RPE represents retinal pigment epithelium.

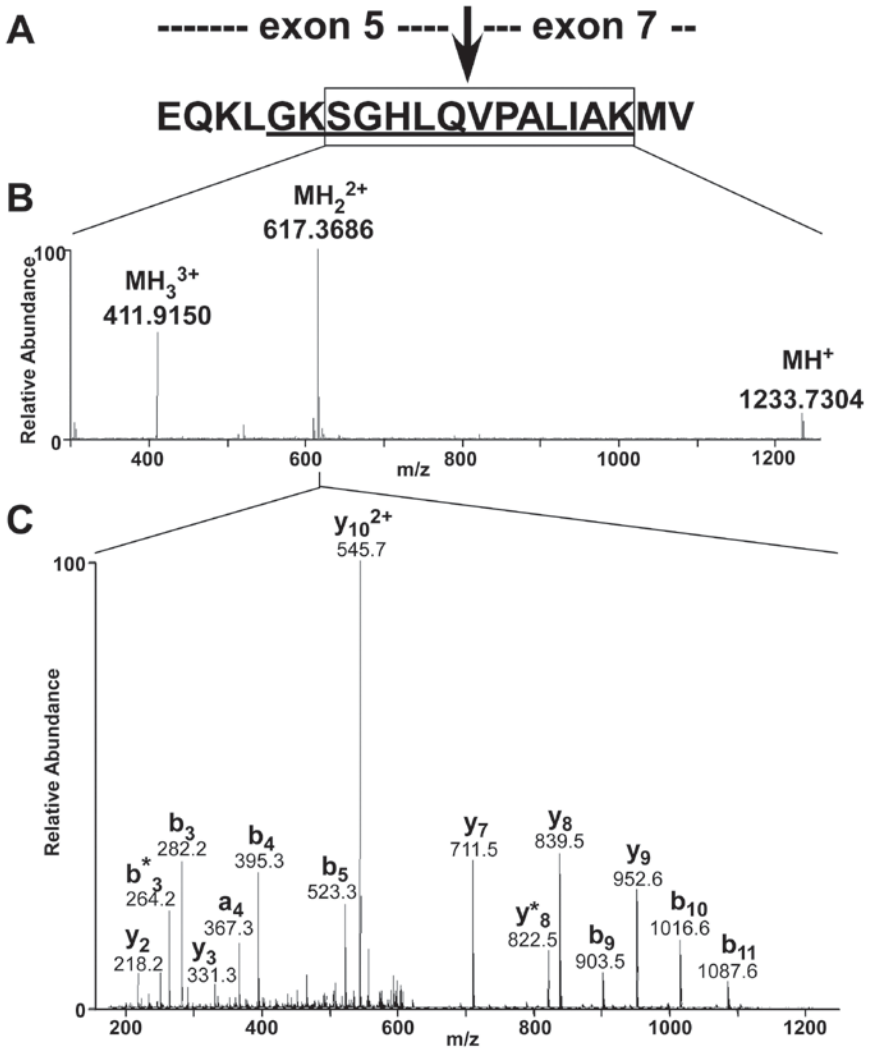

Figure 3. Mass spectrometric analysis of a unique retinal G protein-coupled receptor splice isoform splice-site peptide. **A**: Sequence of a test peptide that encompasses the splice junction site of exons 5 and 7 in retinal G protein-coupled receptor spilce isoform (RGR-d). The peptide contained a tryptic fragment (boxed sequence) and the RGR-d peptide epitope (underlined sequence) used to generate RGR-d specific antibodies. The test peptide was digested with trypsin and characterized by liquid chromatography-mass spectrometry (LC/MS/MS) analysis. **B**: High resolution mass spectrum for the RGR-d splice-site peptide, a tryptic fragment from the test peptide. Ions in the spectrum were annotated to show the charge state and the observed m/z values. **C**: fragment ion spectrum (MS/MS) spectrum for the RGR-d splice-site peptide. Prominent sequence-related ions in the spectrum are annotated to show the observed m/z value and fragment ion series assignment.

We next analyzed retina and RPE membrane proteins from younger individuals (ages 18-21; Figure 2). DE17 reacted with the about 24-kDa RGR-d band and several other bands in the retina of each donor (Figure 2A). In the RPE, however, DE17 reacted primarily with the 18-kDa band, while the other noted bands were barely detectable. As expected, normal RGR was easily detectable in all RPE and retina samples by the HRGR-DE7 antibody (Figure 2B). For each donor, the ratio of intensity of RGR-d to RGR bands was much lower in the RPE in comparison to that in the retina (Figure 2C).

Mass spectrometric analysis of a unique retinal G protein-coupled receptor splice isoform epitope: The RGR-d splice isoform has a contiguous amino acid sequence at the splice junction of exons 5 and 7 that can be detected by our RGR-d antibodies. We next developed an independent method to detect the RGR-d epitope in human tissue by LC/MS/MS analysis. We synthesized a test peptide that contained a tryptic fragment with 12 amino acids of the junction sequence (SGHLQVPALIAK) and corresponded well to the RGR-d epitope (Figure 3A). The test peptide was digested with trypsin,

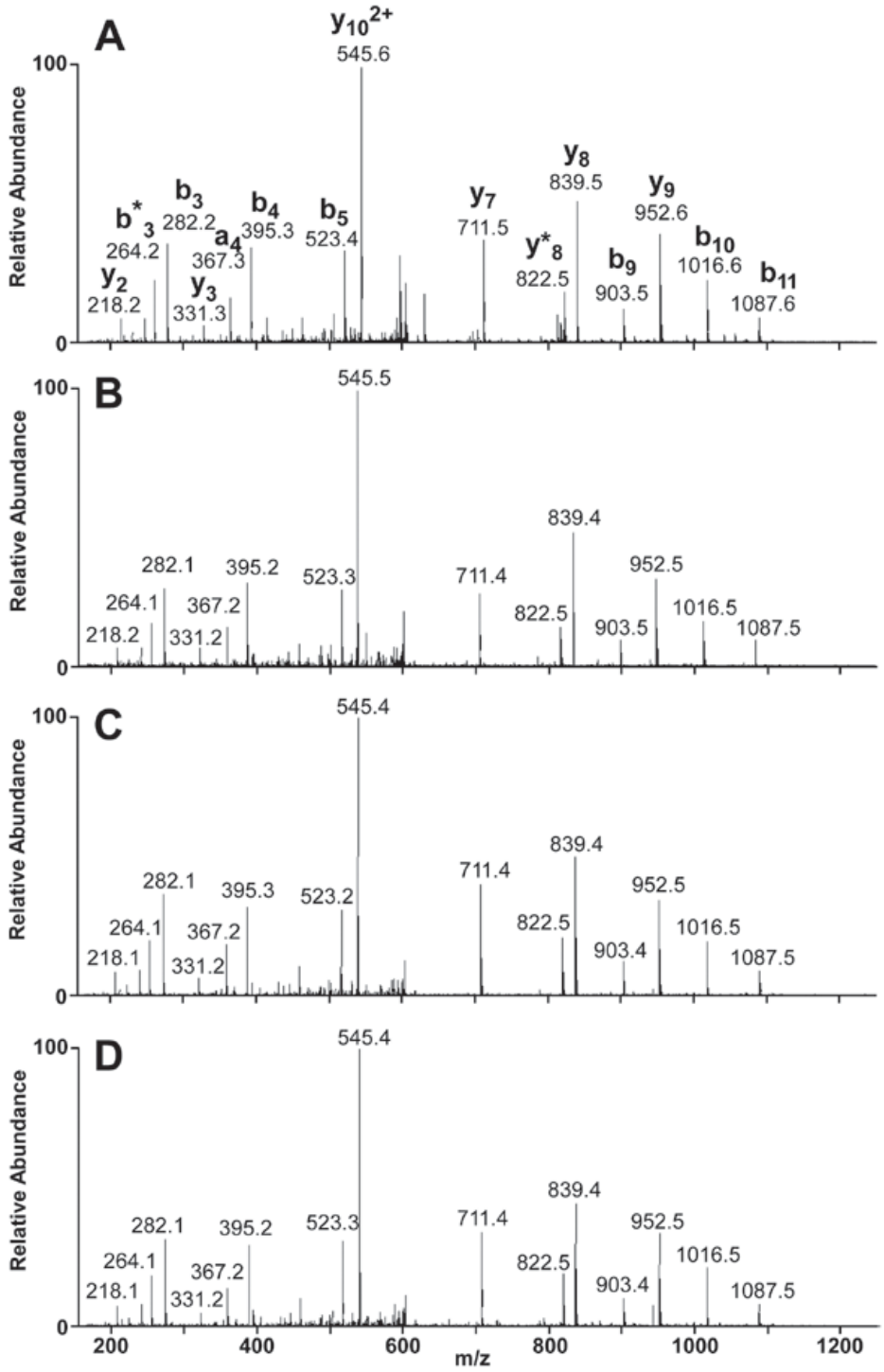

Figure 5. fragment ion spectrum spectra corresponding to the retinal G protein-coupled receptor splice isoform splice-site peptide. Protein bands, which correspond to the immunoreactive bands in the western blot of Figure 4, were stained with Coomassie blue, excised, and analyzed by liquid chromatography-mass spectrometry (LC/MS/MS). Fragment ion spectrum (MS/MS) spectra from precursor ions at m/z 613.4 matching the spectrum for the RGR-d splice-site peptide were observed for (**A**) recombinant RGR-d, (**B**) the about 24-kDa band from donor CA-066, (**C**) the about 55-kDa band from donor CA-066, and (**D**) the about 24-kDa band from donor O. For the spectra from the donor samples, background subtraction was used to eliminate low levels of constant chemical noise evident in the MS/MS spectra obtained at the precursor m/z, mass-to-charge ratio.

and a portion of the reaction was analyzed by LC/MS/MS. We observed a strong peak (Obsv.) with mass values in excellent agreement with those calculated (Calc.) for three charge states of the predicted tryptic fragment (MH+ Calc. 1233.7314, Obsv. 1233.7304, MH_2^{2+} Calc. 617.3693 Obsv. 617.3686, MH_3^{3+} Calc. 411.9153, Obsv. 411.9150; Figure 3B). The peptide assignment was confirmed by a fragment ion spectrum (MS/MS spectrum; Figure 3C) of the 2+ charge state that yielded nearly complete sequence coverage.

Identification of RGR-d in human tissue by mass spectrometric analysis: The recombinant RGR-d, neural retina proteins from donor O and RPE proteins from donor CA-066 were separated by SDS-PAGE and analyzed by western immunoblot (Figure 4). Immunoreactive protein bands (about 24-kDa band from donor O, about 24-kDa and about 55-kDa bands from donor CA-066, and about 24-kDa recombinant RGR-d) were digested with trypsin and subjected to LC/MS/MS analysis. Fragment ion spectra corresponding to the splice-site peptide were recorded for recombinant RGR-d and all three immunoreactive protein bands from the donor samples (Figure 5A-D). In each instance, the spectra were nearly identical to that obtained previously for the test peptide (compare with Figure 3C). To show that a peptide spectrum was not the result of carryover from a preceding run, we performed blank analyses between the samples. These blanks did not yield the correct MS/MS spectrum.

Determination of retinal G protein-coupled receptor splice isoform mRNA by cDNA amplification: Although RGR-d is present in the RPE of donor CA-066, there appears to be little RGR-d protein in the RPE of the other individuals, despite high expression of normal RGR (Figure 1 and Figure 2). It is possible that the RGR-d mRNA was not present in the RPE in these cases. The RGR-d mRNA transcript in donor Y-005 was analyzed, by using RT-PCR to assay the splice variant in the retina and RPE. DNA fragments of about 600 and about 500 nucleotides were amplified from both the retina and RPE. These bands corresponded to the expected fragments of 609 and 495 nucleotides in length for RGR and RGR-d, respectively (Figure 6). No PCR bands were detected in the controls. The about 600- and about 500-bp PCR bands were sequenced directly, and we confirmed their correspondence to the nucleotide sequence of RGR and RGR-d, respectively.

Real-time reverse transcriptase polymerase chain reaction analysis: We also analyzed RGR and RGR-d mRNA by real-time RT-PCR assay. Specific primer-probe sets were designed to amplify RGR or RGR-d cDNA. The downstream primer for full-length RGR corresponds to an exon 6 sequence, so it can not anneal to RGR-d cDNA. The upstream primer for RGR-d spans the junction sequence between exon 5 and 7 and has a mismatch with full-length RGR cDNA of two nucleotides at its 3'-end. Under the PCR conditions, the upstream primer for RGR-d will not anneal to exon 7 nor intiate priming on exon 6 of normal RGR cDNA. The PCR efficiencies of

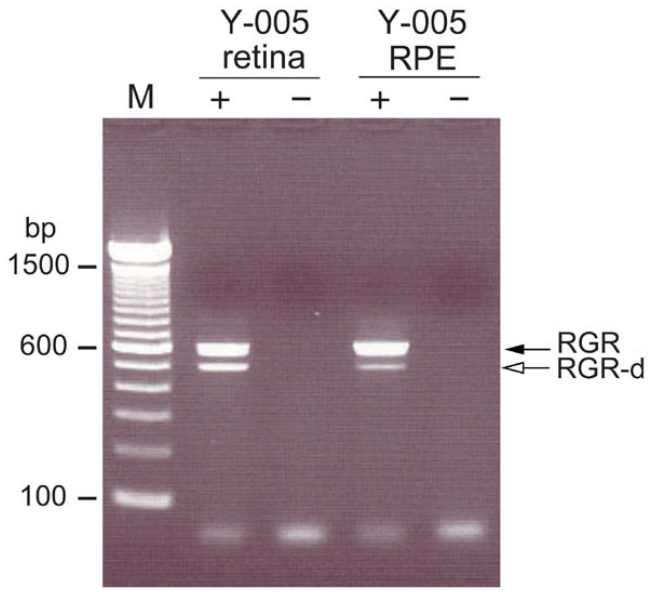

Figure 6. Reverse transcriptase polymerase chain reaction analysis of retinal G protein-coupled receptor mRNA transcripts in the retina and retinal pigment epithelium of donor Y-005. The PCR primers, S200 and A860, were added to first-strand cDNA from the neural retina or retinal pigment epithelium (RPE) of donor Y-005 (OD). Amplification was carried out to generate expected 609-bp and 495-bp fragments that correspond to the intact RGR and truncated RGR-d mRNAs, respectively. Parallel experiments were performed with (+) or without (-) reverse transcriptase. No PCR bands were detected in the controls. The about 600-bp (solid arrow) and about 500-bp (open arrow) PCR fragments from the RPE were sequenced and found to contain the 114-bp presence or deletion of exon 6, respectively.

Table 2. Real-time reverse transcriptase polymerase chain reaction analysis of retinal G protein-coupled receptor and retinal G protein-coupled receptor splice isoform transcripts in the retina and retinal pigment epithelium of donor Y-005

	Cp (mean±SEM)			Expression ratio		Copy number
Tissue	RGR	RGR-d	GAPDH	RGR	RGR-d	RGR-d/RGR
RPE	32.63±0.05	33.87±0.09	35.02±0.19	28.4	15.5	0.17
Retina	30.10±0.06	30.54±0.05	27.61±0.02	1	1	0.33

The relative expression ratios were determined by the method of Pfaffl [27]. Cp values were obtained by the LightCycler 480 software 1.2.0.0625, according to the second derivative maximum method. Abbreviations: RPE represents retinal pigment epithelium, RGR represents retinal G protein-coupled receptor, GAPDH represents glyceraldehyde-3-phosphate dehydrogenase.

the primer-probe sets for RGR, RGR-d and GAPDH were 89.6%, 94.9%, and 95.5%, respectively. The relative expression ratios for the two transcripts were analyzed using guidelines described by Pfaffl [27]. GAPDH was used as the reference in normalization, and the ratios in RPE were based on the expression level in the retina (calibrator).

We analyzed the same total RNA from donor Y-005, as in Figure 6. Normal intact RGR mRNA was about 28 fold higher in the RPE than in the retina (Table 2). This result is consistent with both western and northern blots that indicate higher expression of RGR in the RPE as compared to the retina [28]. The RGR-d splice isoform was also higher by about 16 fold in the RPE than in the retina. Both the end-point and real-time PCR assays confirm that the exon-skipping RGR-d mRNA is expressed in human RPE. The ratio of RGR-d to RGR transcript copy number was 0.17 in the RPE and 0.33 in the retina. These results show that the difference in mRNA transcript ratios between the RPE and retina does not correspond to the much larger difference in RGR-d protein between the two tissues from donor Y-005 (Figure 2).

Immunohistochemical localization of retinal G protein-coupled receptor splice isoform in Bruch's membrane: Our results indicate that although the RPE cells of donor Y-005 have a relatively high amount of the RGR-d mRNA, there is a negligible amount of the RGR-d protein, which also was barely detected in the RPE cells of several other donors (Figure 1 and Figure 2). To further investigate RGR-d in the RPE, we analyzed the RPE of donor Y-005 and other individuals by

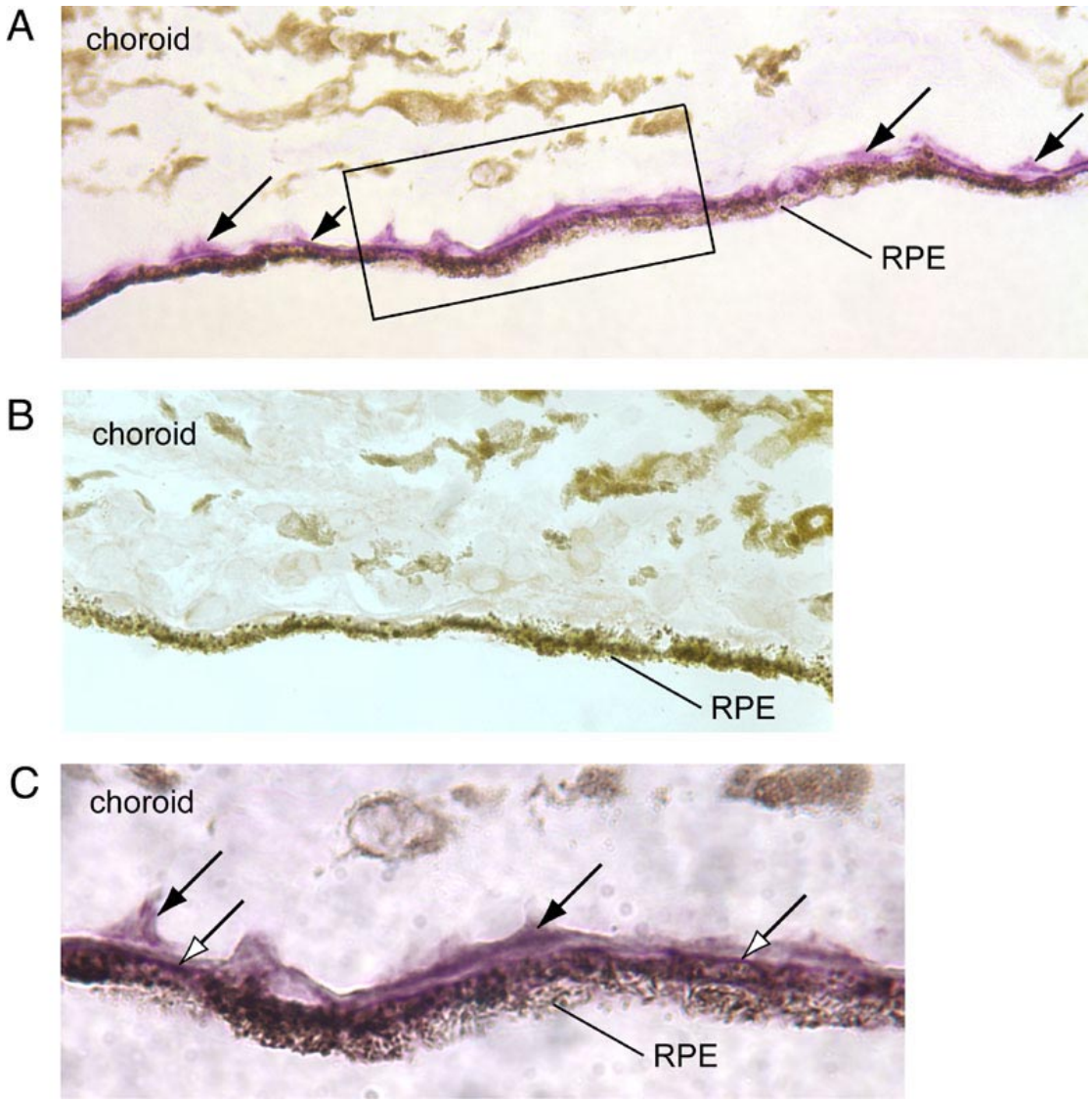

Figure 7. Immunohistochemical localization of the retinal G protein-coupled receptor splice isoform epitope in Bruch's membrane. **A**: The retina or retinal pigment epithelium (RPE)-choroid section was obtained from the macular region of donor Y-005 (OD) and incubated with the DE21 antibody. Arrows point to immunostaining with the VIP substrate in Bruch's membrane and intercapillary region of the choriocapillaris. **B**: Control RPE-choroid section incubated with peptide-blocked DE21 antibody. **C**: Higher magnification of boxed region in A, showing immunostaining of intercapillary regions (solid arrows) and basal boundary of RPE cells (open arrows). The neural retina was detached and absent from the section.

immunohistochemical staining. Tissue sections from donors Y-005 (20/F), CA-0022 (77/F), and 960105 (69/M) were incubated with RGR-d antibody DE21.

Immunostaining of the RPE-choroid in donor Y-005 yielded a relatively weak signal in the RPE cells; however, there was significant positive staining of Bruch's membrane (Figure 7). The RGR-d epitope was present well into the intercapillary region (Figure 7A). Specific immunostaining was blocked by pre-incubation of the antibody with excess peptide (Figure 7B). At higher magnification, any signal in the RPE appeared to be localized to the extreme basal boundary of the cell (Figure 7C).

In donor CA-0022, the RGR-d epitope was present in the intercapillary region, Bruch's membrane and drusen (Figure 8). The immunostaining of the drusen was strong and extensive. On the other hand, immunostaining of RPE cells of this donor was weak or undetectable. Background immunostaining in the choroid was negligible.

Immunostaining of the neural retina of donor 960105 showed that RGR-d is localized in the Müller cells (Figure 9). The Müller cell somata, endfeet and processes revealed strong immunostaining (Figure 9A). Positive staining also appeared along the basal side of the RPE in Bruch's membrane and the intercapillary region (Figure 9B). Positive staining within the RPE was difficult to detect, although there clearly was no staining in the apical portion of the RPE cells. Donor 960105 had macular degeneration and a large subretinal scar in the macula [23].

DISCUSSION

There is now ample evidence for the expression of the RGR-d splice isoform in human eyes. As much as 44% of RGR cDNA clones in a human retina cDNA library encode the RGR-d variant [13]. The RGR-d mRNA is detectable in all samples of donor retinas examined thus far by RT-PCR amplification. An antibody that is directed against an RGR-d-specific peptide binds preferentially to a protein consistent in size to the splice isoform in human retinas [23]. Mass spectrometric analyses of proteins that correspond to the immunoreactive bands independently confirm the presence of the RGR-d splice junction in human neural retina and, in some cases, RPE cells. Unlike other nonfunctional mRNAs, the RGR-d transcript leads to the constitutive production of the encoded protein. Synthesis of the splice isoform would preclude the stoichiometric expression of normal RGR, a protein that is necessary to maintain normal levels of 11-*cis*-retinal after exposure to light [18-20]. Hence, overexpression of RGR-d may affect dark adaptation in humans.

We previously showed that the abundance of RGR-d in the retina varied between individual donors. Here, we show by a more sensitive western blot assay that the RGR-d protein is detectable in the neural retina of each donor as bands of about 24 and about 55 kDa on the blot. The about 24-kDa and about 55-kDa bands appear to be the monomer and an aggregate of the RGR-d protein, respectively. Confirmation of these bands as immunoreactive forms of RGR-d is strongly supported by mass spectrometric analysis and identification of the splice junction peptide, which corresponds to the expected tryptic fragment of RGR-d. The analysis of young and older donors suggests that all individuals have some RGR-d in the neural retina.

We also showed that there is a large difference in the relative amount of RGR-d protein between tissues, the neural retina and RPE cells. The RGR-d/RGR protein ratio is consistently

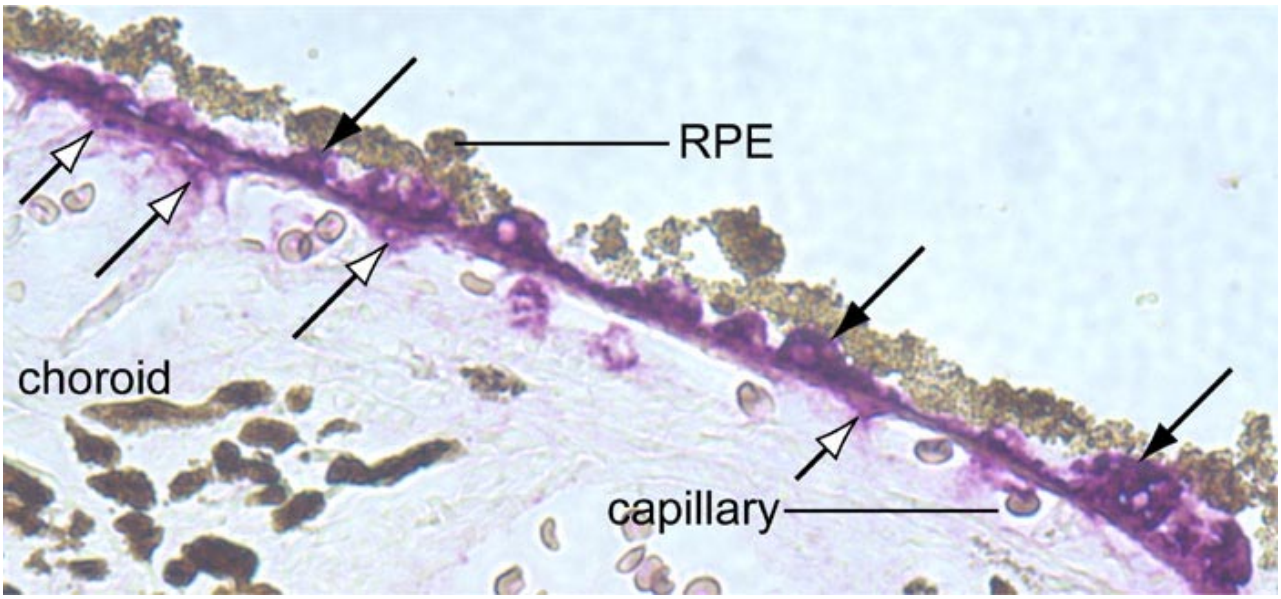

Figure 8. Immunohistochemical localization of retinal G protein-coupled receptor splice isoform epitope in the drusen of donor CA-0022. The retinal pigment epithelium-choroid section from a 77-year-old female donor was prepared from frozen tissue and incubated with the DE21 antibody. The purple VIP chromogen indicates positive staining of drusen (solid arrows), Bruch's membrane, and adjacent areas of the choriocapillaris (open arrows). The neural retina was detached (not shown).

lower in the RPE than in the retina among all donors. Surprisingly, the RGR-d protein in the RPE is nearly undetectable in many cases, despite abundant expression of normal RGR (Figure 1 and Figure 2). High levels of the splice isoform in the RPE appear to be the exception, as in donor CA-066 (Figure 1A). The retina of the young donors contained a few additional high-MW RGR-d bands. The reason for these other bands is unknown, but all the high-MW RGR-d bands were dramatically lower in the RPE than in the retina.

The lack of RGR-d protein in the RPE is not due to the absence of the RGR-d mRNA transcript. The RGR-d mRNA is clearly present in RPE cells as determined by real-time RT-PCR analysis. Both RGR-d and RGR mRNA were higher in the RPE than in the neural retina. In donor Y-005, RGR-d mRNA was present at a rather high percentage of normal RGR mRNA, i.e. 17% and 33% of the amount of normal RGR in the RPE and retina, respectively. The 33% proportion in the retina may seem startling, but it is not inconsistent with our previous finding that as much as 44% of RGR-hybridizing cDNA clones in a human retina cDNA library encodes the RGR-d variant [13]. The RGR-d transcript is stable enough to allow ready cloning of ample cDNAs [13] and is clearly translatable in both mammalian and insect cells [23]. Thus, it is an enigma for the RGR-d mRNA, but not the RGR-d protein to be found in the RPE, especially since the RGR-d protein is expressed in the neural retina of the same individual.

The low abundance of RGR-d protein in the RPE of many donors was corroborated by immunohistochemical analysis of tissue sections. Our results showed relatively weak or little immunostaining within RPE cells and were consistent with western blot assays. Instead, we observed specific positive immunostaining of extracellular regions near the basal aspect of the RPE, including Bruch's membrane and intercapillary region. This finding indicated that a greater amount of the RGR-d epitope was located outside of the RPE cells than inside. The extracellular staining on the basal side of the RPE was seen previously with the strong DE21 antibody [23]. We have also observed extracellular staining with the weaker DE17 antibody, although the results were not as convincing because of the lower signal intensity or poor tissue morphology [23]. Immunostaining for normal RGR in the RPE by the HRGR-DE7 antibody was consistently intense. In the neural retina, RGR-d was localized in the Müller cells.

Since RGR-d is synthesized in the RPE, the extracellular localization of the RGR-d epitope suggests that the splice variant is released or exocytosed at the basal cell surface. The epitope appears to be a residual processed form of RGR-d, which lacks the carboxyl terminus, since it is not immunoreactive with the HRGR-DE7 antibody. The mechanism by which RGR-d is processed in the RPE and deposited into the extracellular space at the basal surface is unknown. The mechanism may involve exocytosis, shedding, blebbing or diffusion from the basal membrane of RPE cells, but it is unlikely to involve RPE cell death in young individuals. The RGR-d isoform may induce its deposition into the extracellular space or enter into an unidentified native pathway for release or exocytosis.

In either case, the RGR-d epitope serves as a strong marker for a type of extracellular deposit beyond the basal margin of human RPE. The synthesis and deposition of RGR-d occurs in young and old throughout adult life and must involve continuous clearance of the epitope. Interestingly, strong positive

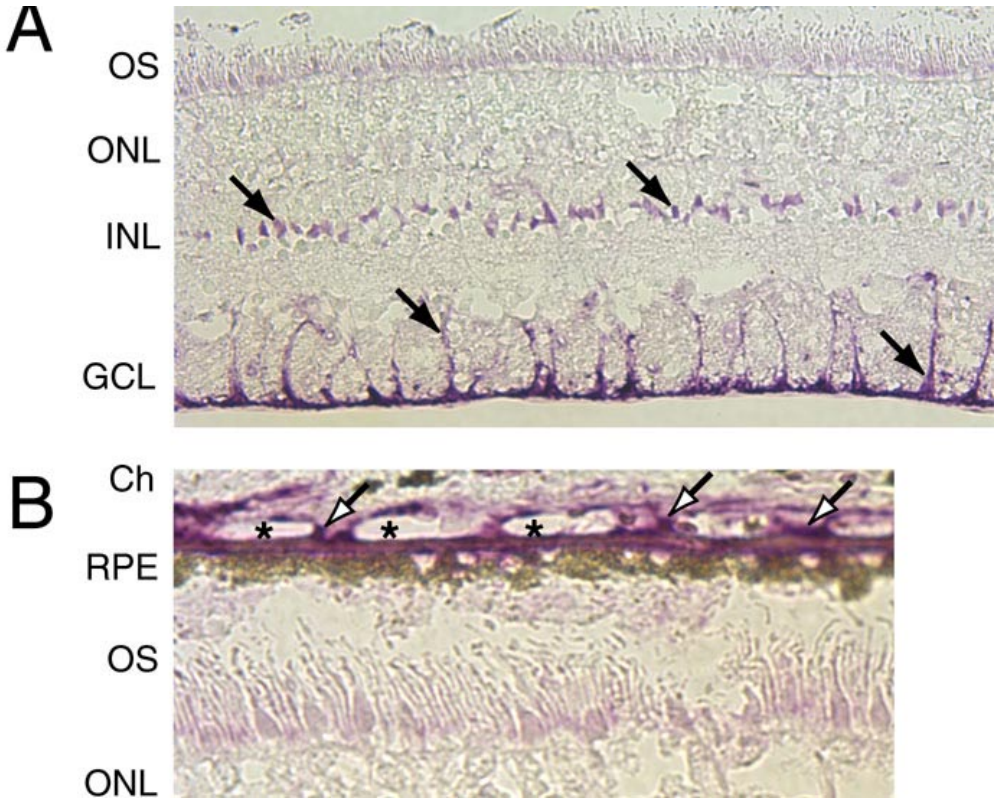

Figure 9. Immunohistochemical localization of the retinal G protein-coupled receptor splice isoform epitope in Müller cells. **A**: The retina section was prepared from the macular region of donor 960105, a 69-year-old male with macular degeneration, and incubated with the DE21 antibody. Positive staining (solid arrows) in the neural retina was evident in the somata and quite strongly, in the inner processes and endfeet of Müller cells. **B**: Higher magnification of positive staining of the intercapillary region (open arrows) and Bruch's membrane. Asterisks indicate lumen of the choriocapillaris. OS represents outer segments; ONL represents outer nuclear layer; INL represents inner nuclear layer; GCL represents ganglion cell layer; Ch represents choroid; RPE represents retina pigment epithelium.

immunostaining of the RGR-d epitope was seen in drusen often found in the older donors. Various other basal extracellular deposits are well known to appear in humans, some as early as 20 years of age [29,30]. It is possible that the extraneous splicing of the human *RGR* gene and continuous expression of RGR-d are associated with some of these deposits and are involved in progressive aging changes in Bruch's membrane, or in the pathogenesis of age-related macular degeneration under certain conditions.

ACKNOWLEDGEMENTS

We thank Mao Yang and Ernesto Barron for valuable technical assistance and the Doheny Eye and Tissue Transplant Bank for providing donor tissue for this research. This work was supported by grants EY03040, EY08364 (HKWF) and CA33752 (TDL) from the U.S. Public Health Service and by an unrestricted grant from Research to Prevent Blindness, Inc.

REFERENCES

1. Boue S, Letunic I, Bork P. Alternative splicing and evolution. Bioessays 2003; 25:1031-4.
2. Mironov AA, Fickett JW, Gelfand MS. Frequent alternative splicing of human genes. Genome Res 1999; 9:1288-93.
3. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, Gibbs RA, Muzny DM, Scherer SE, Bouck JB, Sodergren EJ, Worley KC, Rives CM, Gorrell JH, Metzker ML, Naylor SL, Kucherlapati RS, Nelson DL, Weinstock GM, Sakaki Y, Fujiyama A, Hattori M, Yada T, Toyoda A, Itoh T, Kawagoe C, Watanabe H, Totoki Y, Taylor T, Weissenbach J, Heilig R, Saurin W, Artiguenave F, Brottier P, Bruls T, Pelletier E, Robert C, Wincker P, Smith DR, Doucette-Stamm L, Rubenfield M, Weinstock K, Lee HM, Dubois J, Rosenthal A, Platzer M, Nyakatura G, Taudien S, Rump A, Yang H, Yu J, Wang J, Huang G, Gu J, Hood L, Rowen L, Madan A, Qin S, Davis RW, Federspiel NA, Abola AP, Proctor MJ, Myers RM, Schmutz J, Dickson M, Grimwood J, Cox DR, Olson MV, Kaul R, Raymond C, Shimizu N, Kawasaki K, Minoshima S, Evans GA, Athanasiou M, Schultz R, Roe BA, Chen F, Pan H, Ramser J, Lehrach H, Reinhardt R, McCombie WR, de la Bastide M, Dedhia N, Blocker H, Hornischer K, Nordsiek G, Agarwala R, Aravind L, Bailey JA, Bateman A, Batzoglou S, Birney E, Bork P, Brown DG, Burge CB, Cerutti L, Chen HC, Church D, Clamp M, Copley RR, Doerks T, Eddy SR, Eichler EE, Furey TS, Galagan J, Gilbert JG, Harmon C, Hayashizaki Y, Haussler D, Hermjakob H, Hokamp K, Jang W, Johnson LS, Jones TA, Kasif S, Kaspryzk A, Kennedy S, Kent WJ, Kitts P, Koonin EV, Korf I, Kulp D, Lancet D, Lowe TM, McLysaght A, Mikkelsen T, Moran JV, Mulder N, Pollara VJ, Ponting CP, Schuler G, Schultz J, Slater G, Smit AF, Stupka E, Szustakowski J, Thierry-Mieg D, Thierry-Mieg J, Wagner L, Wallis J, Wheeler R, Williams A, Wolf YI, Wolfe KH, Yang SP, Yeh RF, Collins F, Guyer MS, Peterson J, Felsenfeld A, Wetterstrand KA, Patrinos A, Morgan MJ, de Jong P, Catanese JJ, Osoegawa K, Shizuya H, Choi S, Chen YJ, Szustakowki J, International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 2001; 409:860-921. Erratum in: Nature 2001; 412(6846):565.
4. Brett D, Hanke J, Lehmann G, Haase S, Delbruck S, Krueger S, Reich J, Bork P. EST comparison indicates 38% of human mRNAs contain possible alternative splice forms. FEBS Lett 2000; 474:83-6.
5. Croft L, Schandorff S, Clark F, Burrage K, Arctander P, Mattick JS. ISIS, the intron information system, reveals the high frequency of alternative splicing in the human genome. Nat Genet 2000; 24:340-1.
6. Kan Z, States D, Gish W. Selecting for functional alternative splices in ESTs. Genome Res 2002; 12:1837-45.
7. Modrek B, Lee C. A genomic view of alternative splicing. Nat Genet 2002; 30:13-9.
8. Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet 1999; 8:1893-900.
9. Hillman RT, Green RE, Brenner SE. An unappreciated role for RNA surveillance. Genome Biol 2004; 5:R8.
10. Graveley BR. Alternative splicing: increasing diversity in the proteomic world. Trends Genet 2001; 17:100-7.
11. Sorek R, Shamir R, Ast G. How prevalent is functional alternative splicing in the human genome? Trends Genet 2004; 20:68-71.
12. Lareau LF, Green RE, Bhatnagar RS, Brenner SE. The evolving roles of alternative splicing. Curr Opin Struct Biol 2004; 14:273-82.
13. Jiang M, Shen D, Tao L, Pandey S, Heller K, Fong HK. Alternative splicing in human retinal mRNA transcripts of an opsin-related protein. Exp Eye Res 1995; 60:401-6.
14. Pandey S, Blanks JC, Spee C, Jiang M, Fong HK. Cytoplasmic retinal localization of an evolutionary homolog of the visual pigments. Exp Eye Res 1994; 58:605-13.
15. Sun H, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. Peropsin, a novel visual pigment-like protein located in the apical microvilli of the retinal pigment epithelium. Proc Natl Acad Sci U S A 1997; 94:9893-8.
16. Hara-Nishimura I, Matsumoto T, Mori H, Nishimura M, Hara R, Hara T. Cloning and nucleotide sequence of cDNA for retinochrome, retinal photoisomerase from the squid retina. FEBS Lett 1990; 271:106-10.
17. Hao W, Fong HK. The endogenous chromophore of retinal G protein-coupled receptor opsin from the pigment epithelium. J Biol Chem 1999; 274:6085-90.
18. Chen P, Hao W, Rife L, Wang XP, Shen D, Chen J, Ogden T, Van Boemel GB, Wu L, Yang M, Fong HK. A photic visual cycle of rhodopsin regeneration is dependent on Rgr. Nat Genet 2001; 28:256-60.
19. Maeda T, Van Hooser JP, Driessen CA, Filipek S, Janssen JJ, Palczewski K. Evaluation of the role of the retinal G protein-coupled receptor (RGR) in the vertebrate retina in vivo. J Neurochem 2003; 85:944-56.

20. Wenzel A, Oberhauser V, Pugh EN Jr, Lamb TD, Grimm C, Samardzija M, Fahl E, Seeliger MW, Reme CE, von Lintig J. The retinal G protein-coupled receptor (RGR) enhances isomerohydrolase activity independent of light. J Biol Chem 2005; 280:29874-84.
21. Morimura H, Saindelle-Ribeaudeau F, Berson EL, Dryja TP. Mutations in RGR, encoding a light-sensitive opsin homologue, in patients with retinitis pigmentosa. Nat Genet 1999; 23:393-4.
22. Shen D, Jiang M, Hao W, Tao L, Salazar M, Fong HK. A human opsin-related gene that encodes a retinaldehyde-binding protein. Biochemistry 1994; 33:13117-25.
23. Fong HK, Lin MY, Pandey S. Exon-skipping variant of RGR opsin in human retina and pigment epithelium. Exp Eye Res 2006; 83:133-40.
24. Sarks SH, Sarks JP. Age-related maculopathy: Nonneovascular age-related macular degeneration and the evolution of geographic atrophy. In: Ryan SJ, editor. Retina, 3rd ed. Vol 2. St. Louis: Mosby Inc; 2001. p. 1064-1099.
25. Guymer R, Bird AC. Age changes in Bruch's membrane and related structures. In: Ryan SJ, editor. Retina, 3rd ed. Vol 2. St. Louis: Mosby Inc; 2001. p. 1051-1063.
26. Hellman U, Wernstedt C, Gonez J, Heldin CH. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem 1995; 224:451-5.
27. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45.
28. Jiang M, Pandey S, Fong HK. An opsin homologue in the retina and pigment epithelium. Invest Ophthalmol Vis Sci 1993; 34:3669-78.
29. Hogan MJ, Alvarado JA, Weddell JE. Histology of the Human Eye. Philadelphia: Saunders; 1971. p. 328-363
30. Feeney-Burns L, Ellersieck MR. Age-related changes in the ultrastructure of Bruch's membrane. Am J Ophthalmol 1985; 100:686-97.

The print version of this article was created on 19 Jul 2007. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.