Coexpression of nonvisual opsin, retinal G protein-coupled receptor, and visual pigments in human and bovine cone photoreceptors

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Purpose: Retinal G protein-coupled receptor (RGR) mRNA is transcribed in the outer nuclear layer of human retinas; however, it is not known whether the *RGR* gene is expressed in the rod or cone photoreceptors. In this study, we investigate broader expression of the normal RGR isoform in photoreceptors of human and bovine retinas.
Methods: We produced and validated a rabbit polyclonal antipeptide antibody (DE15) that is directed against a peptide sequence (SSLLRRWPHGSEGC) partly conserved in RGR across several species. Bovine and human retina sections were analyzed with immunohistochemical and double-label immunofluorescent staining.
Results: The DE15 antibody bound specifically to overexpressed recombinant RGR, purified RGR from bovine RPE, and RGR in crude RPE membrane extracts without cross-reaction to other proteins. Immunostaining of diurnal bovine and human retinas with DE15 showed labeling of long-wavelength-sensitive and short-wavelength-sensitive cone photoreceptors and some retinal ganglion cells in both species. Strong labeling with DE15 was detected throughout the cone photoreceptor, including the outer segment, inner segment, cell body, axon, and cone pedicle, while rod outer segments were negative. Immunostaining for human exon-6-skipping RGR (RGR-d) was found primarily at the tips of the outer segment of the cones.
Conclusions: The results indicate that the cone photoreceptors in these mammals express a nonvisual opsin of the Go/RGR or tetraopsin group. RGR and the visual pigments are predominantly colocalized in the cone outer segment, which suggests functional interaction among these opsins. Human cone photoreceptors may also contain normal RGR and the aberrant RGR-d splice isoform.

The *RGR* gene (OMIM 600342), and the genes that encode Go-coupled opsins, peropsin, neuropsin, and retinochrome, belong to the group of tetraopsins (also, Group 4 or Go/RGR opsins), one of the major opsin families [1-3]. Along with the r-opsins (rhabdomeric) and c-opsins (ciliary), tetraopsins are present in all Bilateria, and orthologs of RGR can be found throughout the subphylum Vertebrata, except marsupials [4]. In mammals that have been analyzed, the *RGR* gene is expressed in the RPE and glial Müller cells of the retina [5-7]. Bovine RGR is bound to all-*trans*-retinal in the dark and forms a pigment that absorbs blue (λ_{max} 469 nm) and near-ultraviolet (UV) light (λ_{max} 370 nm) [8]. The chromophore is photoisomerized to 11-*cis*-retinal, rather than 13-*cis*-retinal [9,10]. In mice, RGR plays a significant role in the retinoid cycle, as this protein is required for production of normal levels of 11-*cis*-retinal and rhodopsin under constant illumination, or in darkness after transient photobleaching

[11-14]. Of medical importance, mutations in the *RGR* gene are implicated in patients with dominantly inherited peripapillary choroidal atrophy (c.824dupG, p.I276Nfs*77) [15]. Although the mutant p.I276Nfs*77 appears to be rare, it underlies a process by which an abnormal membranous protein in the RPE can have dominant effects on the choroid and patients' visual ability. More common aberrant splicing of RGR pre-mRNA in human eyes results in an exon-6-skipping isoform, referred to as RGR-d [16]. RGR-d, or a fragment thereof, has been detected in the RPE basolateral plasma membrane, Bruch's membrane, Müller cells, and in older donors, in hard and soft drusen and the subcapillary region of the choroid [17-20].

Tetraopsins and visual pigments are coexpressed in the photoreceptor cells of divergent species, that is, *Platynereis*, *Todarodes pacificus*, and the *Terebratalia transversa* embryo [21-28]. In the human retina, but not in the mouse retina, RGR mRNA has been detected reproducibly in the outer nuclear photoreceptor layer with in situ hybridization with non-overlapping RNA probes [29]. We also have observed that cone outer segments in retina sections from human donors are immunoreactive to an antipeptide antibody that is directed

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against a unique splice junction sequence of RGR-d. If RGR-d is present in human cones, it may be that the *RGR* gene is normally expressed in these neurons in humans and other vertebrates. In this study, we investigated the wider expression of RGR opsin in mammalian photoreceptors.

METHODS

Antibodies: We produced and authenticated rabbit polyclonal antipeptide antibodies against RGR. Synthetic peptides, and peptides conjugated to keyhole limpet hemocyanin for immunization, were obtained from the Caltech Biopolymer Synthesis Center (Caltech, Pasadena, CA). Rabbit antisera were generated by Cocalico Biologicals, Inc. (Reamstown, PA). The antibodies were purified with affinity chromatography using peptide immobilized to Affi-Gel 10 resin (Bio-Rad Laboratories, Hercules, CA). The DE15 antibody is directed against a peptide sequence (SSLLRRWPHGSEGC) that is partly conserved in RGR across several different species. The DE1 antibody was generated against a synthetic peptide (CLSPQRREHSREQ) that corresponds to the carboxyl terminus of bovine RGR [5]. The RGR-d-specific

antibody, DE21, was generated against a synthetic peptide (GKSGHLQVPALIAK) that corresponds to the unique sequence of human RGR-d at the splice junction of exons 5 and 7 [17]. DE21 immunoreactivity was validated by the ability of the antibody to specifically bind recombinant human RGR-d protein [17]. The cone opsin antibodies, OPN1MW/LW (sc-22117) and OPN1SW (sc-14363), were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Tissue preparation: All experiments and procedures were conducted in compliance with the applicable regulatory guidelines at the University of Southern California, the principles of human research subject protection in the Declaration of Helsinki, and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects. Postmortem eyes were obtained from the Doheny Eye and Tissue Transplant Bank (Los Angeles, CA) and the National Disease Research Interchange (NDRI, Philadelphia, PA). Bovine eyes were obtained from a local abattoir. Tissues were dissected from the central retina and fixed with 4% paraformaldehyde in PBS (D-5652; Sigma-Aldrich, St. Louis, MO) for 4–6 h at 4 °C. Fixed tissues were infiltrated overnight with 30%
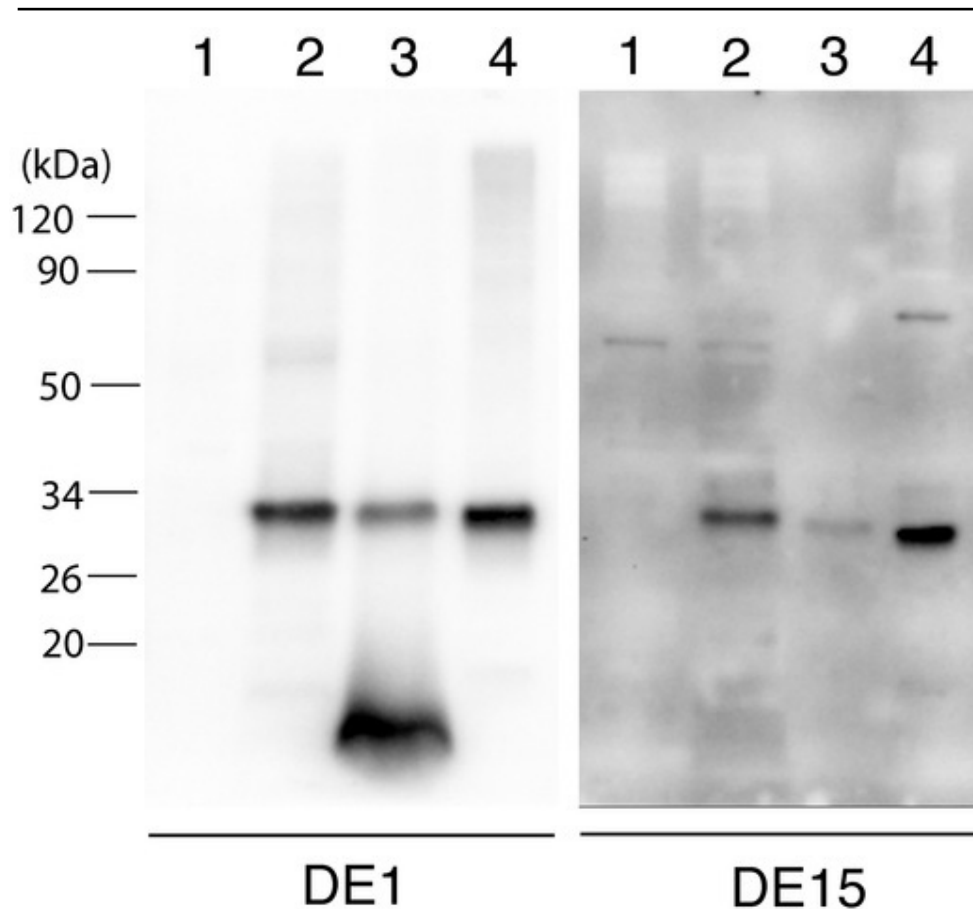

Figure 1. Authentication of specific binding of DE15 antibody to bovine RGR. The immunoreactivity of affinity-purified antibodies DE1 (*left panel*) and DE15 (*right panel*) was tested with an immunoblot assay of various preparations of bovine RGR protein. (**Lane 1**) Control untreated Sf9 cell extract. (**Lane 2**) Bovine RGR baculovirus-transduced Sf9 cell extract. (**Lane 3**) Purified RGR from bovine RPE. (**Lane 4**) Bovine RPE microsomal membranes. The protein blots were incubated with DE1 or DE15 antibodies, and binding was detected with chemiluminescence.

sucrose in PBS. The retina/RPE/choroid complex was removed from the sclera and embedded in optimum cutting temperature (OCT) compound (Miles, Elkhart, IN) before freezing. The frozen tissues were sectioned with a cryostat at −20 °C to a thickness of 5–8 μm and mounted on Superfrost Plus slides (Thermo Fisher Scientific, Pittsburgh, PA). Tissue blocks and slides were stored at −80 °C.

Immunohistochemistry: Bovine and human eye tissue sections were analyzed with immunohistochemical staining with affinity-purified primary antibodies. The sections were permeabilized with fixation with cold acetone for 5 min. The samples were then incubated with blocking buffer that consisted of 0.2% dodecyl maltoside, 3% BSA (BSA), and 5% normal goat serum in PBS. After blocking, the sections were incubated with primary antibody diluted in 0.2% dodecyl maltoside in PBS. Immunohistochemical staining was performed with the ImmPRESS peroxidase-based enzyme detection system and Vector VIP substrate (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. Control slides were treated in the same manner, except that the primary antibody was omitted from the incubation buffer. The sections were dehydrated sequentially with 95% and 100% ethanol, cleared with xylene, and covered with VectaMount Permanent Mounting Medium (Vector Laboratories). Images were photographed using the Aperio Scanscope Model CS (Leica Biosystems, Buffalo Grove, IL).

Immunofluorescent staining: Double-label immunofluorescence was performed with sequential incubation of tissue sections with different sets of primary antibody and fluorochrome-conjugated secondary antibody. Frozen sections were thawed and incubated first with affinity-purified DE15 and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody (FI-1000; Vector Laboratories). For double-labeling, the sections were washed with 0.1% Tween in PBS and incubated with OPN1MW/LW or OPN1SW cone opsin primary antibodies, and Alexa Fluor 568-conjugated donkey anti-goat secondary antibody (A-11057; Life Technologies Corp., Foster City, CA). The sections were dehydrated and mounted with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were obtained with a PerkinElmer 6-line spinning disk laser confocal microscope (PerkinElmer, Waltham, MA).

Western immunoblot assay: Recombinant bovine RGR protein was produced with the Bac-to-Bac Baculovirus
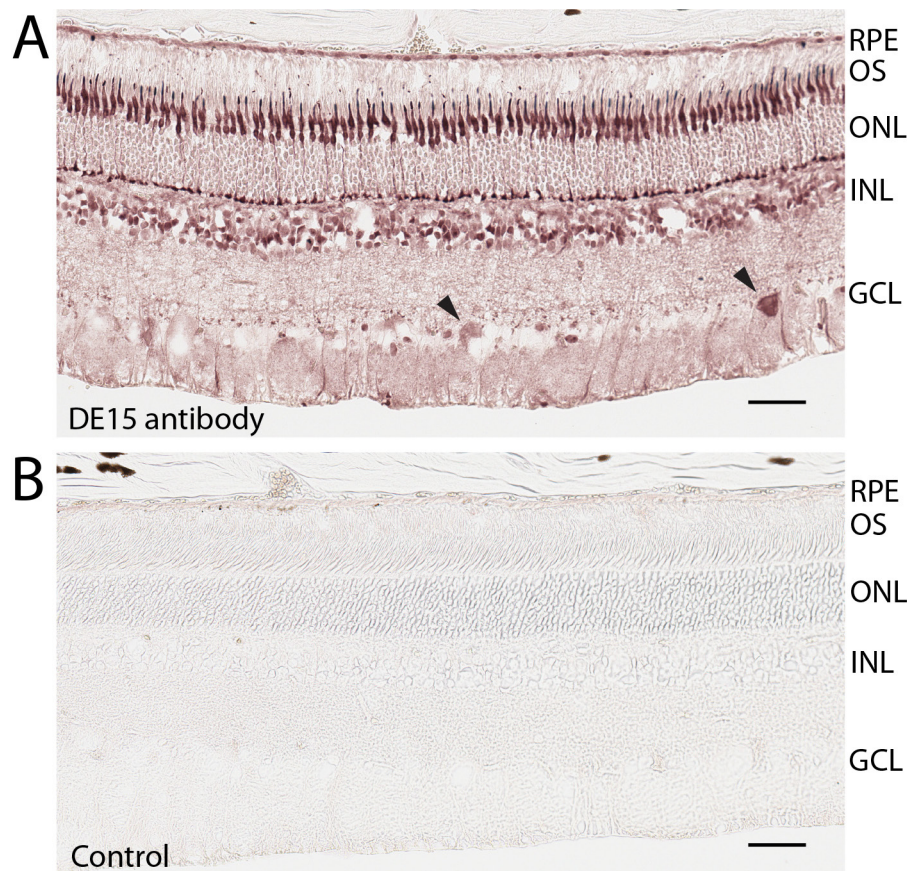

Figure 2. RGR opsin in bovine cone photoreceptors. **A**: Retinal tissue and frozen sections were prepared from fresh bovine eyes after fixation. The tissue section was incubated with affinity-purified DE15 antibody, and immunostaining was detected with the Vector VIP peroxidase substrate. Neuronal immunostaining was detected throughout the cone photoreceptor and in a few retinal ganglion cells (arrowheads). Non-neuronal immunostaining was in the RPE and in Müller cell bodies and processes. **B**: The negative control slide was treated in parallel, except that the primary antibody was omitted from the incubation buffer. No counterstains were used. RPE, retinal pigment epithelium; OS, outer segment layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 50 μm.

Expression System (Invitrogen, Carlsbad, CA), according to the manufacturer's procedures. RGR was isolated from bovine RPE, as described previously [8]. Briefly, RPE cells were removed from bovine eyecups by gently scraping the cell monolayer with a spatula. Microsomal membranes were isolated and twice extracted for 1 h at 4 °C with 1.2% digitonin (Eastman Kodak Co., Rochester, NY) in 10 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl and 0.5 mM EDTA. After centrifugation of the extract at 100,000 × *g* for 20 min, RGR was isolated from the supernatant by immunoaffinity binding to anti-bovine RGR

monoclonal antibody 2F4. RGR was eluted with wash buffer that contained 100 µM bovine RGR carboxyl-terminal peptide (CLSPQRREHSREQ). Bovine RPE microsomal membranes were prepared, as described previously [30]. RPE cells were collected by centrifugation and homogenized in ice-cold buffer of 250 mM sucrose, 30 mM sodium phosphate pH 6.5, using a Dounce glass homogenizer. The homogenate was centrifuged at 700 × *g* at 4 °C to remove nuclei and unbroken cells. The supernatants from the homogenization steps were centrifuged in a Sorvall SS-34 rotor at 15,000 × *g* for 20 min at 4 °C. The 15,000 × *g* supernatant contained the
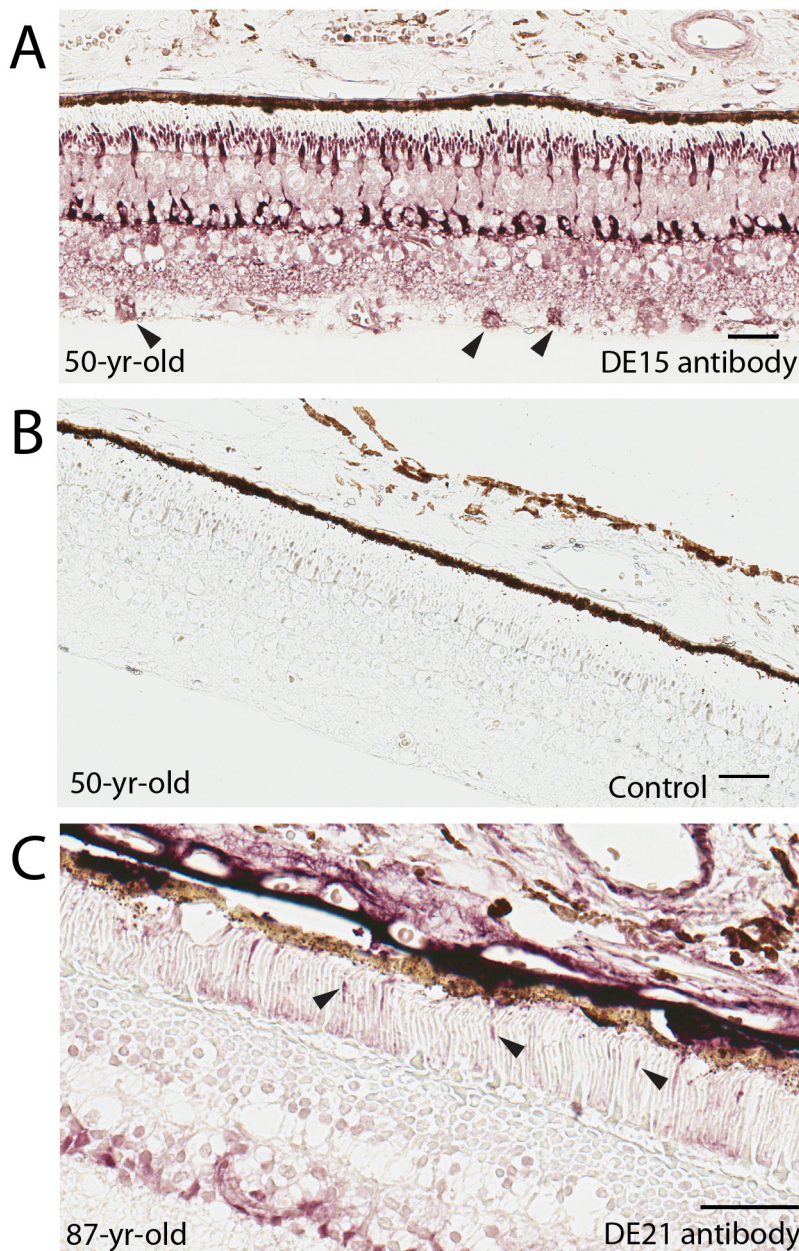

Figure 3. RGR opsin in human cone photoreceptors. **A**: RGR opsin in cone photoreceptors of a 50-year-old human donor. Retinal sections were prepared from tissue fixed in 4% paraformaldehyde and embedded in frozen optimum cutting temperature (OCT) compound. The sections were probed with the DE15 antibody. The tissue section contained an intact retina still attached to the choroid. A few retinal ganglion cells (arrowheads) showed positive immunostaining for RGR. **B**: The negative control slide was treated in parallel as in *panel A*, except that the DE15 primary antibody was omitted from the incubation buffer. **C**: Immunohistochemical staining of RGR-d in cone photoreceptor outer segments of an 87-year-old donor. The sections were probed with affinity-purified DE21 antibody, which is directed against human RGR-d. The section contained the intact retina attached to the RPE layer and choroid. RGR-d was seen in Bruch's membrane, intercapillary regions, subcapillary regions, drusen and basal deposits, Müller cell bodies, and cone photoreceptor outer segments (arrowheads). No counterstains were used. Scale bars, 50 µm.
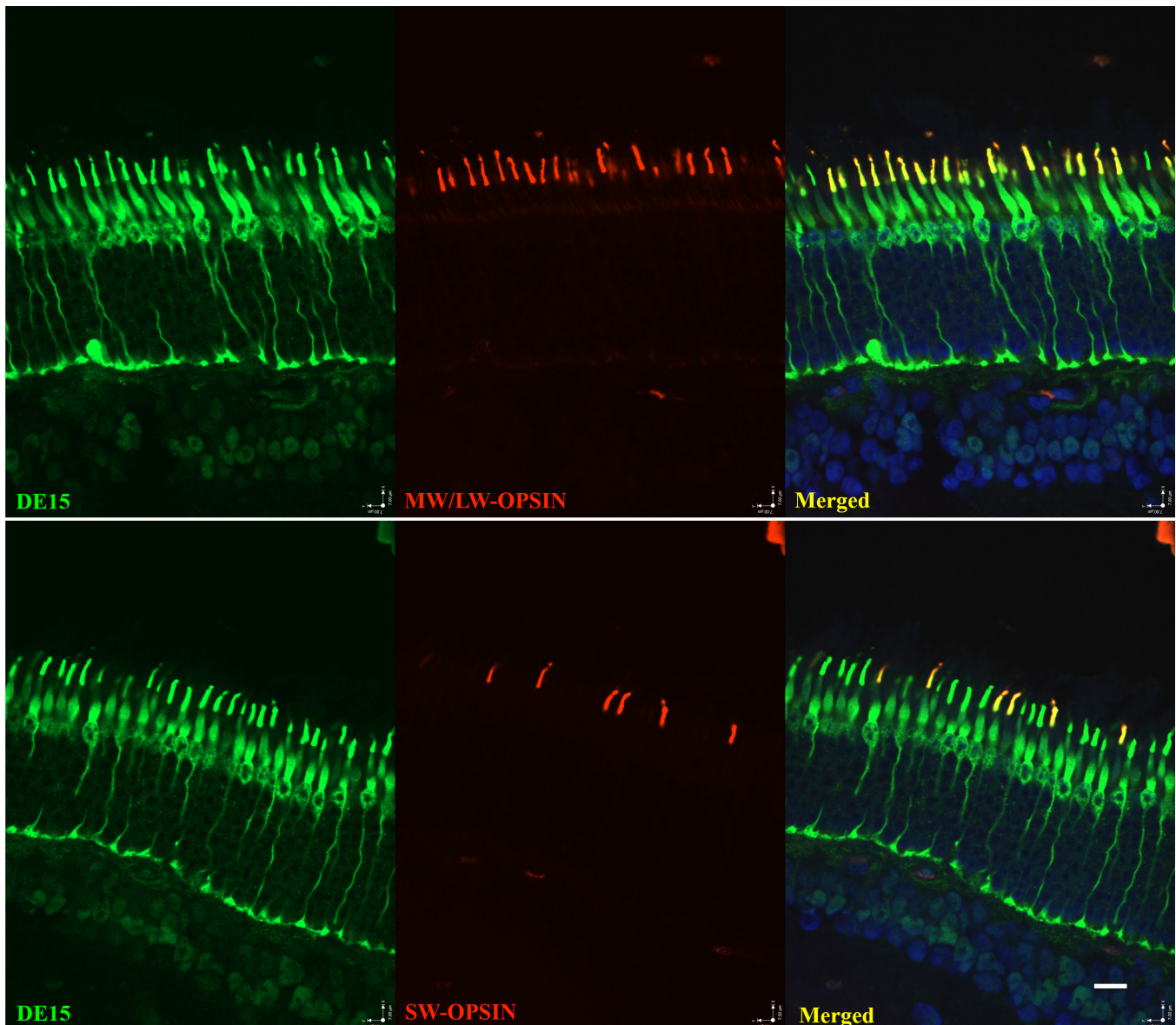
Figure 4. Colocalization of bovine RGR and cone opsins with double-label immunofluorescence. RGR and cone visual pigments were detected with DE15 (RGR), and OPN1MW/LW or OPN1SW opsin antibodies, respectively. The sections were incubated first with affinity-purified DE15 and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody. For double-labeling, the sections were washed and incubated with the OPN1MW/LW (*top panel*), or OPN1SW (*bottom panel*) primary antibody and Alexa Fluor 568-conjugated donkey anti-goat secondary antibody. Images were obtained with a PerkinElmer 6-line spinning disk laser confocal microscope. The merged images with 4',6-diamidino-2-phenylindole (DAPI) counterstain showed intense labeling and colocalization of RGR and cone visual pigment in the outer segments of both types of bovine cone photoreceptors. Scale bar, 25 µm.

RPE microsome fraction from which the membranes were then collected by centrifugation in a Beckman 70 Ti rotor at 150,000 × *g* for 1 h at 4 °C. The pellet was resuspended in 10 mM sodium phosphate pH 6.5, 150 mM NaCl, 0.5 mM EDTA, and 20% glycerol buffer.

Recombinant bovine RGR protein, purified RGR, and membrane protein extracts from bovine RPE cells were analyzed with Western immunoblotting. Proteins were denatured and reduced in Laemmli sample buffer, resolved by electrophoresis in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels (25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS), and then transferred to 0.2-µm Immuno-Blot polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). The blots were blocked by incubation in 5%

non-fat milk in PBS, probed with affinity-purified primary antibody at ambient temperature, washed, and incubated with a secondary antibody that was conjugated to horseradish peroxidase. Primary and secondary antibodies were suspended in 0.1% Tween-20 in PBS. Membrane washes were performed using 0.1% Tween-20/PBS. Immunoreactive antigens were detected with chemiluminescence using the horseradish peroxidase–based enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) system or the SuperSignal West Femto (Pierce Biotechnology, Rockford, IL) substrate system. Chemiluminescence was detected and documented with the Bio-Rad ChemiDoc XRS+ Gel Imaging System (Bio-Rad Laboratories).

RESULTS

Antibody validation: RGR mRNA has been detected in the outer nuclear photoreceptor layer in the human retina, but not in the mouse retina, with in situ hybridization [29]. It may be that the *RGR* gene is actively expressed in the photoreceptors of some species, notwithstanding the usual finding that carboxyl terminal antibodies against RGR do not appear to immunostain photoreceptors. It is possible that immunostaining of the carboxyl terminal epitope of RGR with anti-peptide antibodies is blocked in these photoreceptors. To probe a different epitope of RGR, we produced and tested another antibody (labeled DE15) that recognizes a peptide sequence (SSLLRRWPHGSEGC) in the first extracellular, or intraluminal, loop of RGR.

The DE15 antibody bound positively to overexpressed recombinant RGR in extracts from baculovirus-transduced Sf9 insect cells but did not react with the protein extracts from untreated control Sf9 cells (Figure 1, *lanes 1 and 2*). This antibody bound positively to purified RGR that was isolated from bovine RPE (Figure 1, *lane 3*). In addition, DE15 bound specifically to native RGR in crude membranes from bovine RPE with little cross-reaction to other proteins (Figure 1, *lane 4*). A specific protein band that corresponds in size to that of RGR (about 32 kDa) was found in each test protein sample but not in the non-transduced control Sf9 cells. DE1 and DE15 reacted similarly in relative binding intensity to recombinant
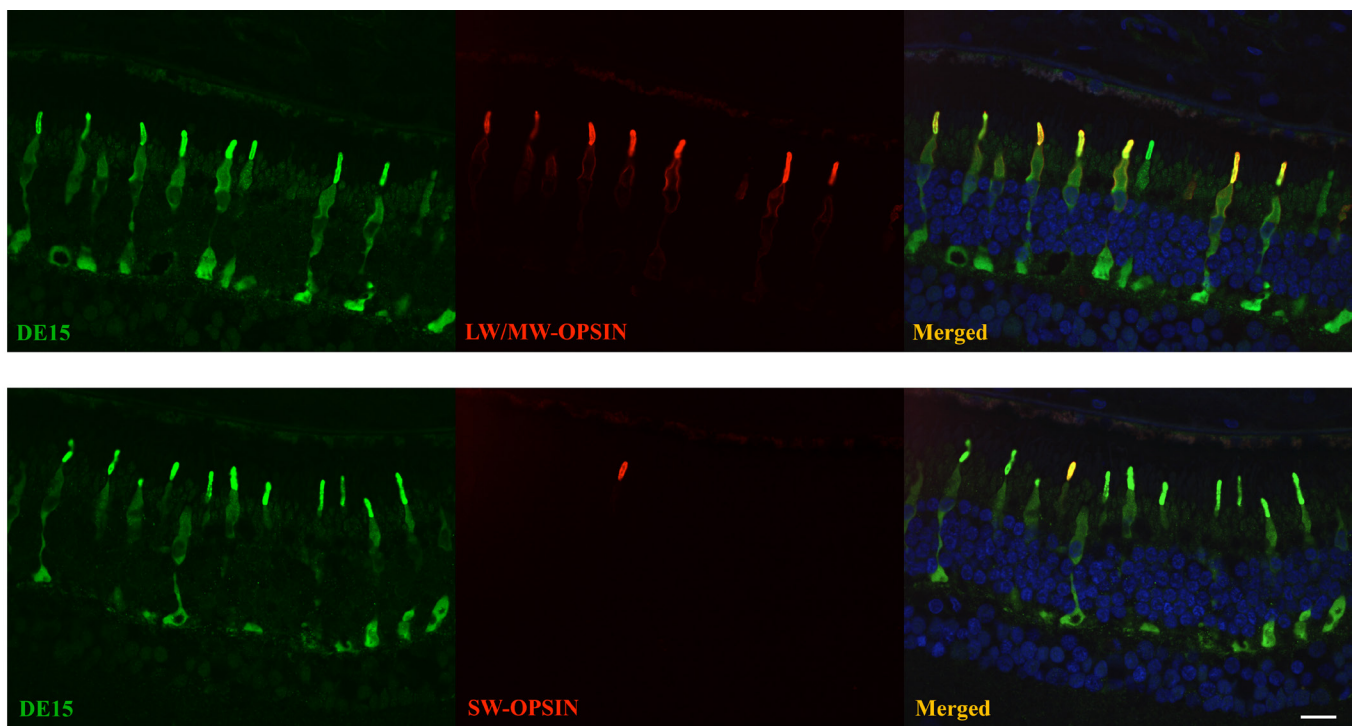

Figure 5. RGR opsin in the red/green and blue cone photoreceptors of a 50-year-old donor. RGR and cone visual pigments were detected with double-label immunofluorescent staining with DE15, and OPN1MW/LW or OPN1SW opsin antibodies, respectively. The sections were probed first with affinity-purified DE15 and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody. Subsequently, the sections were incubated with the OPN1MW/LW (*top panel*), or OPN1SW (*bottom panel*) primary antibody and Alexa Fluor 568-conjugated donkey anti-goat secondary antibody. The images with 4',6-diamidino-2-phenylindole (DAPI) counterstain showed intense labeling that revealed coexpression of RGR and cone visual pigment in the outer segments of all red/green and blue cone photoreceptors. RGR and cone opsins were colocalized also in the inner segments of red/green cone photoreceptors. Scale bar, 25 μm.

bovine RGR, purified RGR protein, and RGR in the crude RPE microsomes.

Immunolocalization of RGR opsin in cone photoreceptors: We used the DE15 antibody to immunostain bovine and human retinas. Immunohistochemical staining with DE15 showed labeling of cone photoreceptors in the bovine (Figure 2) and 50-year-old (C/M) human donor (Figure 3A,B) retinas. Strong labeling with DE15 was detected throughout the cone photoreceptor, including the outer segment, inner segment, cell body, axon, and synaptic terminal. A few retinal ganglion cells were also positive in the bovine and human retinas. The area that had nonpigmented RPE in the bovine retina allowed easier visualization of positive RPE staining. As the epitope of RGR is within the lumen of the endoplasmic reticulum, the DE15 antibody is immunoreactive in RPE, but immuno-labeling may be weakened by poor epitope availability.

RGR-d isoform in human cone outer segments: We found that the cone outer segments in the retina sections from human donors were also immunoreactive to an antipeptide antibody (DE21) that is directed against the exon-6-skipping isoform of the human retinal G protein-coupled receptor (RGR-d; Figure 3C). Immunostaining for human RGR-d was promi-nent in the cone outer segments in some donors, while other donors showed little or no cone RGR-d. In an 87-year-old donor (C/F), overall RGR-d immunoreactivity was strongest in Bruch's membrane, intercapillary regions, subcapillary boundaries, and drusen (Figure 3C). RGR-d was also detected in Müller cell bodies in the inner retina of this donor.

RGR opsin in distinct types of cone photoreceptors: We performed double-label immunofluorescence to identify the type of cone photoreceptor that expresses RGR opsin. Bovine dichromatic color vision is based on separate cones that have a short-wavelength-sensitive or long-wavelength-sensitive opsin [31]. RGR was found in each type of bovine cone photoreceptor and colocalized with the visual pigment in the cone outer segment (Figure 4).

In the human retina, RGR was present in all green-/red-sensitive cones, as well as in blue-sensitive cones (Figure 5). RGR and visual pigments colocalized in the cone outer segment, but the overall distribution of the antigens was different. RGR, but not the cone opsins, distributed also to the synaptic ends of the cone photoreceptor. The differential staining pattern indicates that the immunoreactivity of the DE15 antibody in cones is not due to cross-reaction by cone opsins.

DISCUSSION

These results indicate that RGR opsin is expressed in cone neurons, the photoreceptors used for bright-light and color vision in bovine and human eyes. Although antibodies that are directed against the carboxyl terminus of RGR do not immunostain cone photoreceptors, at least two independent antibodies, DE15 and DE21, detect RGR isoforms in cone outer segments. We authenticated the DE15 antibody, which localizes RGR in the cone outer segment, inner segment, cell body, axon, and synaptic terminal. This widespread cellular distribution is similar to the localization of cone proteins that are involved in signaling, that is, the G protein β_3 subunit [32], γ_8 subunit [33], and cone arrestin [34]. The DE21 antibody localizes the human RGR-d isoform primarily in the outer segment of the cone photoreceptor with possible variation in the amount or processing of the RGR-d protein among individuals. The expression of RGR opsin in human cone photoreceptors is consistent with and supported by in situ hybridization, as described previously [29]. Despite several attempts, we could not demonstrate DE15 immunostaining of cone photoreceptors in the mouse retina, which is also in keeping with negative results for in situ hybridization, as reported by Trifunovi et al. [29].

Go/RGR opsins are present in the photoreceptors of other species [21-28]. In *Platynereis* larvae, a Go-opsin (*Platynereis* Go-opsin1) is coexpressed with two r-opsins in the rhabdomeric photoreceptor cells and is involved in phototaxis of the larva [21]. In cephalopods, retinochrome is colocalized with visual pigment in photoreceptor cells and acts directly to regenerate the 11-*cis*-retinal chromophore with photoisomer-ization [35-37]. Possibly, RGR is involved in the regeneration of 11-*cis*-retinal and cone photopigments by regulation of an intraphotoreceptor retinoid cycle or direct photoisomeriza-tion of all-*trans*-retinal via a retinochrome-like mechanism. Thus far, there is scarce evidence of an intracone retinoid cycle in diurnal mammals. The RPE65 isomerohydrolase has been detected in human red/green cones [38] and in cone photoreceptors of other vertebrates [39,40]. Other visual cycle proteins, such as CRALBP, have not been observed in cone photoreceptors. RGR is expressed in the intrinsically photosensitive retinal ganglion cells of the chicken retina and is involved in retinoid metabolism in these neurons, as reported previously [41]. We also showed that RGR is present in a population of retinal ganglion cells in bovine and human retinas (Figure 2 and Figure 3).

Although RGR is distributed throughout the cone photo-receptor, immunolabeling is especially strong in the cone outer segments, the singular function of which is visual photo-transduction. In close proximity to the cone visual pigment,

irradiated RGR may be able to exchange 11-*cis*-retinal with an all-*trans* chromophore of the cone pigment within the hydrophobic membrane of the outer segment. Sorting of RGR to the outer segment membrane would require different targeting signals than that to the endoplasmic reticulum of RPE and may involve modification of the carboxyl terminus of cone RGR. Further biochemical investigation of how RGR may interact with the visual pigment and traffic within cones will be aided greatly by isolation of cone outer segment membranes that contain the RGR opsin.

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