Expression of X-Linked Retinoschisis Protein RS1 in Photoreceptor and Bipolar Cells

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Purpose. To examine the biochemical properties, cell expression, and localization of RS1, the product of the gene responsible for X-linked juvenile retinoschisis.

Methods. *Rs1h* mRNA expression was measured from the eyes of wild-type and *rd/rd* mice by Northern blot analysis and reverse transcription–polymerase chain reaction (RT-PCR). Specific antibodies raised against the N terminus of RS1 were used as probes to examine the properties and distribution of RS1 in retina, retinal cell cultures, and transfected COS-1 cells by Western blot analysis and immunofluorescence microscopy.

Results. *Rs1h* mRNA expression was detected in the retina of postnatal day (P)11 and adult CD1 mice, but not homozygous *rd/rd* mice by Northern blot analysis. However, *Rs1h* expression was detected in *rd/rd* mice by RT-PCR. RS1 migrated as a single 24-kDa polypeptide under disulfide-reducing conditions and a larger complex (>95 kDa) under nonreducing conditions in the membrane fraction of retinal tissue homogenates and transfected COS-1 cells. RS1 antibodies specifically stained rod and cone photoreceptors and most bipolar cells, but not Müller cells, ganglion cells, or the inner limiting membrane of adult and developing retina as revealed in double-labeling studies. RS1 antibodies also labeled retinal bipolar cells of photoreceptorless mice and retinal bipolar cells grown in cell culture.

Conclusions. RS1 is expressed and assembled in photoreceptors of the outer retina and bipolar cells of the inner retina as a disulfide-linked oligomeric protein complex. The secreted complex associates with the surface of these cells, where it may function as a cell adhesion protein to maintain the integrity of the central and peripheral retina. (*Invest Ophthalmol Vis Sci.* 2001;42:816–825)

X-linked juvenile retinoschisis is a recessively inherited, bilateral vitreoretinal degeneration that affects males early in life.[1–3] Affected persons typically experience mild to severe loss of visual acuity in the first decade of life, followed by progressive atrophy of the macula in the mid to later years. Approximately 50% of the patients also have a decrease in visual field. Electroretinograms (ERGs) of most affected persons exhibit near normal a-waves characteristic of photoreceptor function but reduced b-waves originating from inner retinal cell activity.[4,5] In contrast, most female carriers are asymptomatic and exhibit normal ERGs.

A characteristic feature of X-linked retinoschisis is the presence of streaks radiating outward from the parafoveal region of the retina. This spoke-like pattern results from cystic cavities that split the inner retina at the level of the nerve fiber and ganglion cell layers.[6–8] Bilateral schisis is also found in the peripheral retina in approximately half of the affected persons. The extracellular space adjacent to the schisis cavity has been reported to contain amorphous material and filaments that merge with Müller cell membranes.[8] Degeneration of photoreceptors and underlying retinal pigment epithelium is also evident in the macula and affected peripheral regions of the retina.

The gene (*RS1,* formerly *XLRS1*) responsible for X-linked juvenile retinoschisis has been identified by positional cloning.[9] It consists of six exons and encodes a 224-amino-acid protein containing a hydrophobic leader sequence with a consensus signal peptidase cleavage site. RS1, also called retinoschisin, consists of a discoidin-like domain that is found in a family of proteins implicated in cell adhesion.[9–14] A spectrum of genetic mutations is found in persons with X-linked retinoschisis. These include missense and nonsense mutations, insertion and deletion mutations, intragenic deletions, and splice-site mutations.[9,15,16]

RS1 expression is restricted to retinal tissue.[9] In situ hybridization studies have further shown that the murine orthologue *Rs1h* is abundantly expressed in photoreceptors.[14]

To begin to define the role of RS1 in retinal cell biology and X-linked juvenile retinoschisis, we have examined the biochemical properties, cellular expression, and localization of RS1 in mammalian retina and cell cultures. We report here that RS1 is assembled as a disulfide-linked oligomeric protein complex that is expressed and secreted from bipolar cells as well as rod and cone photoreceptors. It interacts with the surface of these cells where it may function as a cell adhesion protein to stabilize the organization of the retina.

METHODS

Animals and Donor Eyes

Balb/c, *rd*, and CD1 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Wistar rats and human donor eyes were obtained from the University of British Columbia animal facilities and eye bank (Vancouver, British Columbia, Canada), respectively. Eyes from a homozygous *rd/rd* cl mouse were the generous gift of Russell G. Foster (Imperial College, London, UK). The care and handling of animals was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RNA Isolation, Northern Blot Analysis, and cDNA Probes

Total RNA was isolated from whole eyes of adult, *rd*/+ and *rd/rd* mice (~8 weeks old) by the guanidinium thiocyanate method.[17] For North-

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ern blot analysis, 12 μg total RNA was run on each lane of a 1.2% agarose gel in the presence of formaldehyde and blotted onto a nylon membrane. A 436-bp cDNA probe (3F6R) representing coding exons 3 through 6 of the murine *Rs1h* gene was amplified using primers rsm3F (5′-TACCTCCTTAGACTGTATTCC-3′) and rsm6R (5′-GATGAAGCGGGAAATGATGG-3′).[13] The murine *Crx* cDNA probe was generated by polymerase chain reaction (PCR) using primers Crx-F (5′-GTCCCCCACCTCCTTGTCAG-3′) and Crx-R (5′-CCTCAAGTTCCCAGCAATCC-3′).[18] A 289-bp murine β-actin probe was generated by PCR using primers XAHR20 (5′-ACCCACACTGTGCCCATCTA-3′) and XAHR17 (5′-CGGAACCGCTCATTGCC-3′; GenBank accession number X03765). The probes were radiolabeled by random priming and consecutively hybridized to the same filter at 65°C in 0.5 mM sodium phosphate buffer (pH 7.2), 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA.[19]

Reverse transcription–polymerase chain reaction (RT-PCR) reactions were performed in a volume of 25 μl. Each reaction contained 1 μl of first-strand cDNA (from whole-eye mouse RNA) as template and 15 picomole primers. PCR conditions were: 94°C, 5 minutes; 94°C, 30 seconds; T_A of each primer pair, 30 seconds; 72°C, 30 seconds; 72°C, 5 minutes. Each PCR was performed for 30 cycles. The primer sequences and reaction conditions were as follows:

- Murine *Rs1h*: rsm3F and rsm6R primers as described earlier. Predicted size: 436 bp; the amplification product spanned exons 3 through 6 of *Rs1h*; PCR conditions: T_A: 59°C; 1.5 mM $MgCl_2$; 4% formamide.
- Opsin: opsin-f primer: 5′-TTCACCACCACCCTCTACAC-3′; opsin-r primer: 5′-GTTGAGGGTGGTCTTGGTGG-3′. Predicted size: 992 bp; GenBank accession number M55171; PCR conditions: T_A: 58°C; 1.5 mM $MgCl_2$.
- β-Actin: XAHR20 and XAHR17 primers were as described earlier. Predicted size: 289 bp; GenBank accession number X03765; PCR conditions: T_A: 58°C; 1.5 mM $MgCl_2$; 4% formamide.

Generation of the RS1 Antibody

A 17-amino-acid peptide (LSSTEDEGEDPWYQKAC) corresponding to amino acids 22-39 of the human RS1 precursor protein[9] was conjugated to keyhole limpet hemocyanin and used to immunize a rabbit. To confirm the specificity of immunolabeling, the RS1 antibody was affinity purified from the antiserum, as previously described.[20] Another RS1 antibody was raised in a mouse immunized with a glutathione-*S*-transferase fusion protein containing the same N-terminal peptide.

Isolation of Retina Tissue and COS-1 Cell Extracts

Retina tissue from mouse or rat eyes was incubated in 400 μl hypotonic buffer (5 mM Tris-HCl [pH 7.4] containing 1 mM Pefabloc SC protease inhibitor; Boehringer-Mannheim, Germany) for 1 hour at 4°C and homogenized in a microfuge tube. The homogenate was layered on 10% sucrose and centrifuged at 26,000 rpm for 20 minutes in a swinging-bucket rotor (model TLS-55; Beckman, Berkeley, CA). The fraction collected above the 10% sucrose solution was defined as the soluble fraction. The pellet resuspended in 100 μl of 10% sucrose was defined as the retinal membrane fraction. Protein concentration was determined by BCA assay (Pierce, Rockford, IL).

Monkey kidney COS-1 cells were transfected with the full-length human *RS1* cDNA in pcDNA3 (Invitrogen, San Diego, CA), as previously described.[21] Cells were homogenized in 10 mM MOPS buffer (3-[*N*-morpholino] propanesulfonic acid; pH 7.5) and separated into a soluble and membrane fraction by centrifugation on a gradient consisting of 40% (wt/wt) and 60% (wt/wt) sucrose.

SDS-PAGE and Western Blot Analysis

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), samples (~3 mg/ml protein) were added to an equal volume of SDS cocktail (125 mM Tris-HCl [pH 6.8], 4% SDS, 40% sucrose, 0.02% bromophenol blue in the presence or absence of 8% β-mercaptoethanol) and run on a 12% polyacrylamide gel. Proteins were transferred onto membranes (Immobilon; Amersham Pharmacia Biotech, Arlington Heights, IL) and Western blots were labeled with the RS1 antiserum diluted 1:3000 in phosphate buffered saline-0.1% Tween 20 (PBS-T) or affinity-purified antibody diluted 1:500 in PBS-T in the presence or absence of competing RS1 peptide, as previously described.[20] The blots were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Baie d'Urfé, Quebec).

Immunofluorescence Labeling

Retina tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 to 2 hours and rinsed in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose. Cryosections were blocked with PBS containing 0.2% Triton X-100 and 10% goat serum for 20 minutes and labeled overnight with the RS1 antiserum or affinity purified antibody diluted 1:2000 in PBS-T containing 2% goat serum. Samples were rinsed in PBS-T and labeled for 1 hour with Alexi 594–conjugated goat anti-rabbit immunoglobulin (Molecular Probes, Eugene, OR). In control samples, sections were labeled with the RS1 antibody in the presence of 0.1 mg/ml RS1 peptide. For double-labeling studies, sections were also labeled with cell-specific monoclonal antibodies (mAbs) and Alexi 488–conjugated goat anti-mouse immunoglobulin. mAbs were: protein kinase C (PKCα) and vimentin (Sigma, St. Louis, MO); PKCβ (Seikagaku America, Falmouth, MA); monoclonal antibody (MAb) 115A10[22] against rat olfactory bulb (ROB; kindly provided by Shinobu C. Fujita, Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan). Polyclonal antibody to cellular retinal binding protein (CRABLP) was a generous gift from Jack Saari (University of Washington, Seattle, WA). Labeled retinal sections were examined under a fluorescence microscope (Axioplan2; Zeiss, Munich, Germany) equipped with a digital image analysis system (Eclipse; Zeiss).

Retinal Cell Cultures

Retinal cell cultures were established as previously described.[23] Briefly, retinas from adult pig eyes were digested with 0.2% papain in PBS for 20 minutes at 37°C, triturated by passage through a Pasteur pipette and seeded at 5×10^5 cells/cm^2 onto laminin-treated coverslips in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 supplemented with 2% fetal calf serum. The medium was refreshed every week. For immunofluorescence microscopy, the cells were fixed in 4% paraformaldehyde, blocked in PBS-T, and incubated overnight with anti-RS1 antiserum diluted 1:3000. After a rinsing in PBS, the cells were permeabilized in 0.1% Triton X-100 and relabeled with retinal-cell–specific antibodies and fluorescence-labeled secondary antibodies, as described.

RESULTS

Rs1h mRNA Expression

Expression of *Rslh* mRNA in adult and developing mouse eye was examined by Northern blot analysis. Two transcripts of 4.9 kb and 5.6 kb were detected in total-eye RNA of postnatal day (P)11 and adult CD1-mice as well as heterozygote adult *rd/+* mice (Fig. 1A). No signal was detected in P0 and P3 CD1 mice and adult homozygous *rd/rd* mice. To test for photoreceptor expression in the developing retina, Northern blots were hybridized with the murine transcription factor *Crx* cDNA probe. It has been shown that *Crx* expression correlates well with the differentiation of the outer plexiform layer at approximately neonatal stage P6 and persists in adult retina.[18] A signal of 3.0 kb was detected in the adult wild-type CD1 mouse and heterozygote *rd/+* mouse but not in the adult homozygous *rd/rd* mouse.

RT-PCR was performed to detect for low *Rs1h* expression. An intense 436-bp product was obtained from wild-type and heterozygous *rd/+* mice retinas, and a weaker product was

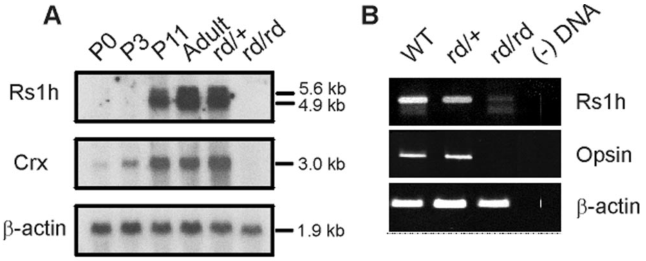

FIGURE 1. *Rs1h* expression in wild-type and *rd* mice. (**A**) Northern blot analysis of total RNA from eyes of P0, P3, P11, and adult CD1-mice as well as heterozygous adult *rd*/+ and homozygous adult *rd*/*rd* mice. Hybridization with an *Rs1h* cDNA probe revealed two distinct transcripts of 4.9 kb and 5.6 kb for P11 and adult CD-1 mice and *rd*/+ mice. No signal was detected for *rd*/*rd* mice. Subsequent hybridization of the same filter with the murine *Crx* probe detected a single transcript at 3.0 kb. RNA integrity and equal loading were assessed with subsequent β-actin control hybridization. (**B**) RT-PCR analysis of *Rs1h*, rod opsin, and β-actin from wild-type CD-1, *rd*/+, and *rd*/*rd* mouse eye cDNA. An intense 436-bp *Rs1h* signal was observed from wild-type (WT) and heterozygous *rd*/+ mice; a weak *Rs1h* signal is observed from homozygous *rd*/*rd* mice in the absence of rod opsin expression. The *band* just below the *Rs1h* product observed in wild-type and *rd*/*rd* samples represents a nonspecific product. Control samples minus DNA were run in parallel.

observed in homozygous *rd*/*rd* mice in the absence of detectable rod opsin expression. Sequence analysis confirmed that the amplified 436-bp product from wild-type and *rd*/*rd* mice was derived from the murine *Rs1* sequence. In addition to the *Rs1h* product, a band just below the 436-bp product was observed in some experiments. Reamplification of this band for sequence analysis was unsuccessful, indicating that it most likely represented a nonspecific product.

Analysis of RS1 in Retinal and Transfected COS-1 Cell Membranes

Antibodies raised against a peptide corresponding to the N terminus of mature RS1 were used to examine the biochemical properties of RS1 in mouse retinal extracts by SDS-PAGE and Western blot analysis. RS1 migrated in the membrane fraction of retinal homogenates as single 24-kDa polypeptide under disulfide-reducing conditions and a large complex (>95 kDa) near the top of the gel under nonreducing conditions (Fig. 2A). No signal was detected in the soluble fraction. In control studies, labeling was abolished when excess competing RS1 peptide was included during the primary-antibody–labeling step (data not shown).

RS1 transiently expressed in monkey kidney COS-1 cells was also analyzed on Western blot analysis. As in the case of retinal homogenates, RS1 migrated in the membrane fraction as a 24-kDa polypeptide under disulfide-reducing conditions and a high-molecular-weight complex under nonreducing conditions (Fig. 2B).

Distribution of RS1 in Mammalian Retina

The distribution of RS1 in mammalian retina was examined by immunofluorescence microscopy. The pattern of outer and inner retina labeling was similar for bovine (Fig. 3), human (Fig. 4 and 5), mouse (Fig. 6), and rat (Fig. 7) and when RS1 antibodies raised in either a rabbit or mouse were used for immunolabeling. Intense staining was observed in the photoreceptor inner segment layer, particularly in the ellipsoid region immediately adjacent to the outer segments. Below the outer limiting membrane, more moderate staining of the photoreceptor cell body and outer plexiform layers was observed. In bovine (Fig. 3) and human retina (Figs. 4, 5), cone inner segment and cell-body staining was outlined against the more abundant rod photoreceptors, a labeling pattern that is consistent with the distribution of RS1 along the surface membrane of cone cells. Weak, diffuse labeling of the photoreceptor outer segment layer was observed in sections containing intact retinal pigment epithelial (RPE) cells (Fig. 3). This labeling most likely represented weak staining of the interphotoreceptor matrix surrounding the photoreceptor outer segments.

The RS1 antibody also stained cells within the inner nuclear layer (INL) of the retina (Figs. 3 through 7). Moderate immunostaining was observed around the cell bodies and more diffuse staining extended from the INL down into the vitreal half of the inner plexiform layer. No staining was observed within the ganglion cell layer (GCL) or along the inner limiting membrane. For comparison, retinal sections were also stained with antibodies to CRABLP and PKCα (Fig. 3). As previously reported,[24] the anti-CRALBP antibody labeled the RPE layer and Müller cells that extend from the outer limiting membrane down through the INL and GCL to the inner limiting membrane. RS1 staining did not resemble CRALBP staining. PKCα antibody stained cell bodies and neurites of rod bipolar cells.[25,26] Some PKCα immunoreactive bipolar cells were double labeled by the RS1 antibody, although the majority of RS1-immunopositive cells were PKCα immunonegative (see also Figs. 5 and 7).

The specificity of RS1 immunolabeling of retinal sections was examined in a series of control studies. The pattern of labeling was the same for antiserum or affinity-purified RS1 antibody and for antibodies raised in a rabbit or mouse. Moreover, addition of excess RS1 peptide during labeling abolished the staining of the photoreceptors and inner retinal cells (see Fig. 6).

RS1 Staining in Human Macula and Peripheral Retina

Because X-linked juvenile retinoschisis affects primarily central vision, we examined the distribution of RS1 within the macula and foveal regions of human retina. The RS1 antibody labeled

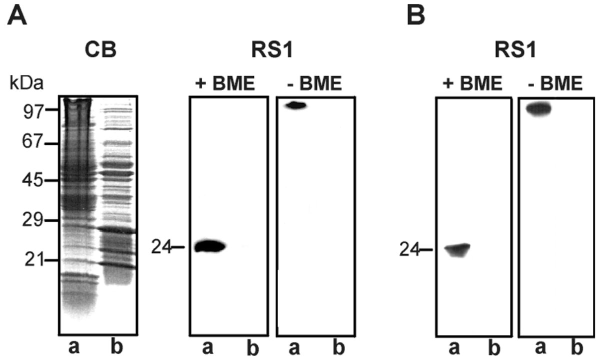

FIGURE 2. SDS polyacrylamide gels and Western blot analysis of mouse retinal extracts and *Rs1h* transfected COS-1 cell homogenates. (**A**) Mouse retina tissue was homogenized in hypotonic buffer, and the membrane fraction was separated from the soluble fraction by high-speed centrifugation. *Left*: SDS gels were run under disulfide-reducing conditions (in the presence of β-mercaptoethanol [BME]) and stained with Coomassie blue (CB); *Right*: Western blot analysis of the reduced (+ BME) and nonreduced (− BME) gels were labeled with the RS1 antibody. (**B**) COS-1 cell homogenate was separated into membrane and soluble fractions. Western blot analysis of reduced (+ BME) and nonreduced (− BME) gels were labeled with the RS1 antibody. In each case, RS1 was found in the membrane fraction and migrated as a 24-kDa protein under reducing conditions and a large complex (>95 kDa) under nonreducing conditions. *Lanes a*: membrane fraction; *lanes b*: soluble fraction.

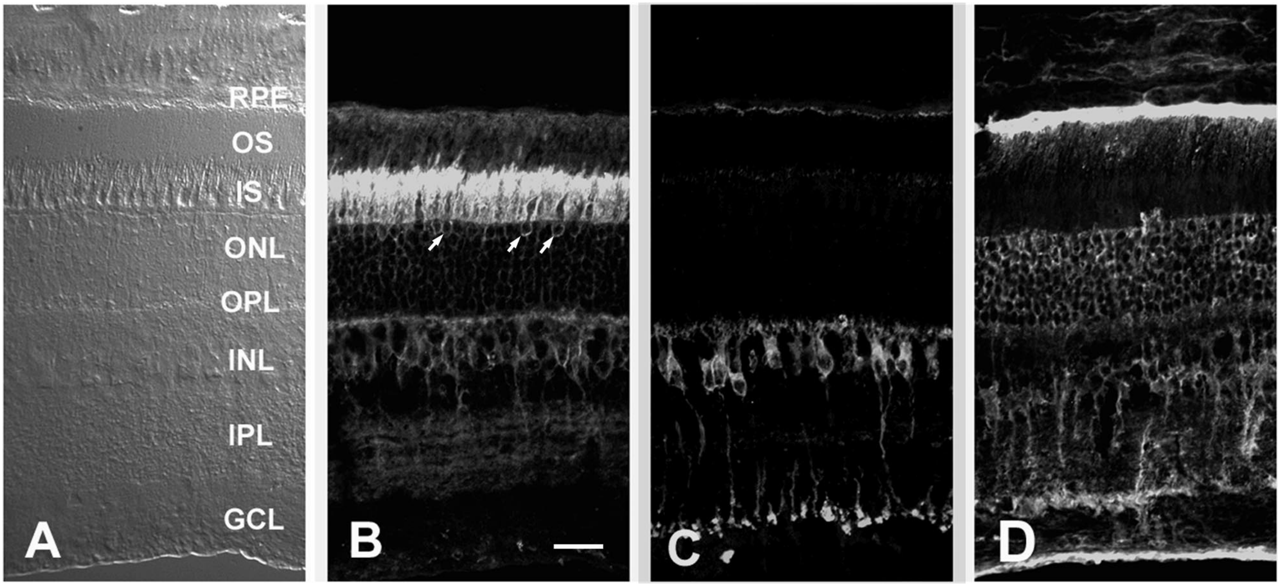

FIGURE 3. Immunofluorescence microscopy of RS1 in bovine retinal tissue. (**A**) Differential interference contrast image showing the various retinal layers: RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer. (**B**) RS1 immunolabeling of outer and inner retina. *Arrows*: outline staining of cone cell bodies. (**C**) PKCα immunolabeling of a subset of rod bipolar cells. (**D**) CRALBP immunolabeling of RPE and Müller cells. Bar, 20 μm.

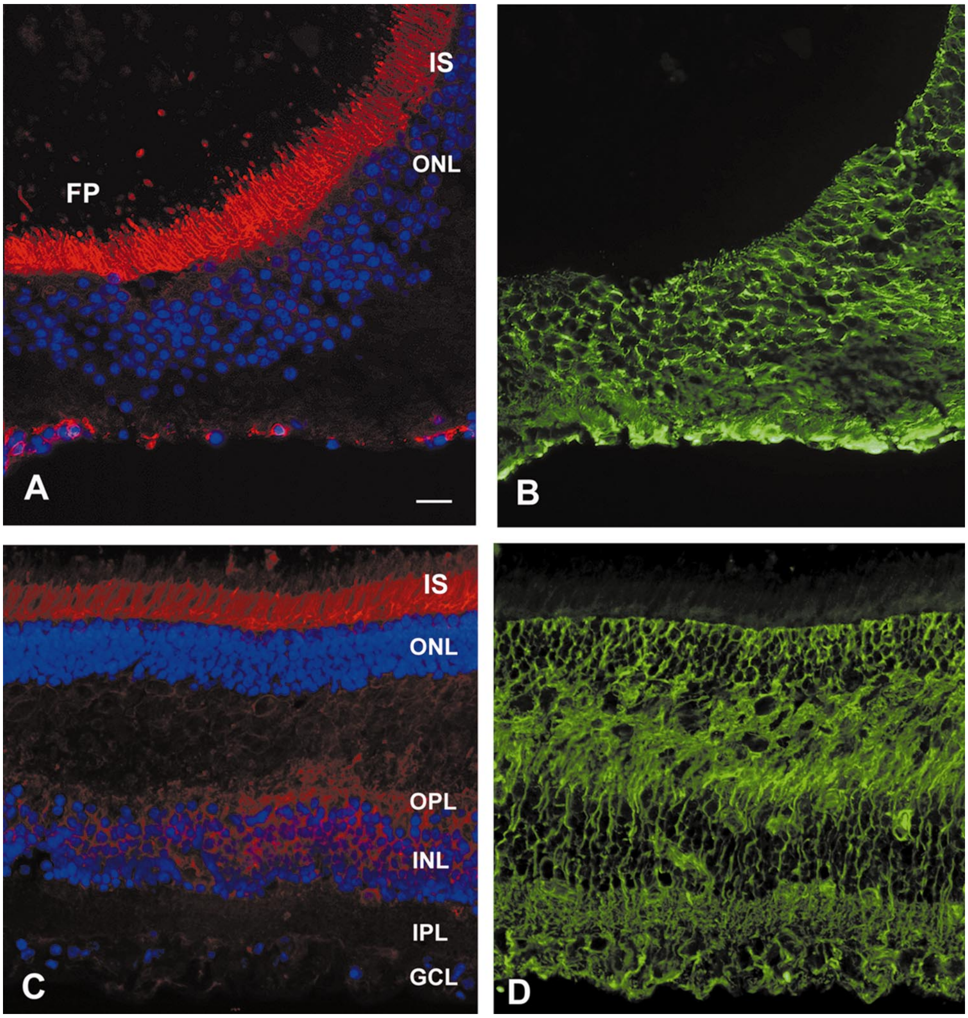

FIGURE 4. Immunofluorescence microscopy of human foveal and macular sections. (**A**) Foveal section labeled with the RS1 antibody (*red*) and 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (*blue*). FP, foveal pit. (**B**) Same section double labeled with anti-vimentin antibody (*green*) specific for Müller cells. (**C**) Macular section outside the fovea labeled with the RS1 antibody (*red*) and DAPI nuclear stain (*blue*). (**D**) Same section double labeled with anti-vimentin antibody (*green*). Abbreviations are defined in Figure 3. Bar, 20 μm.

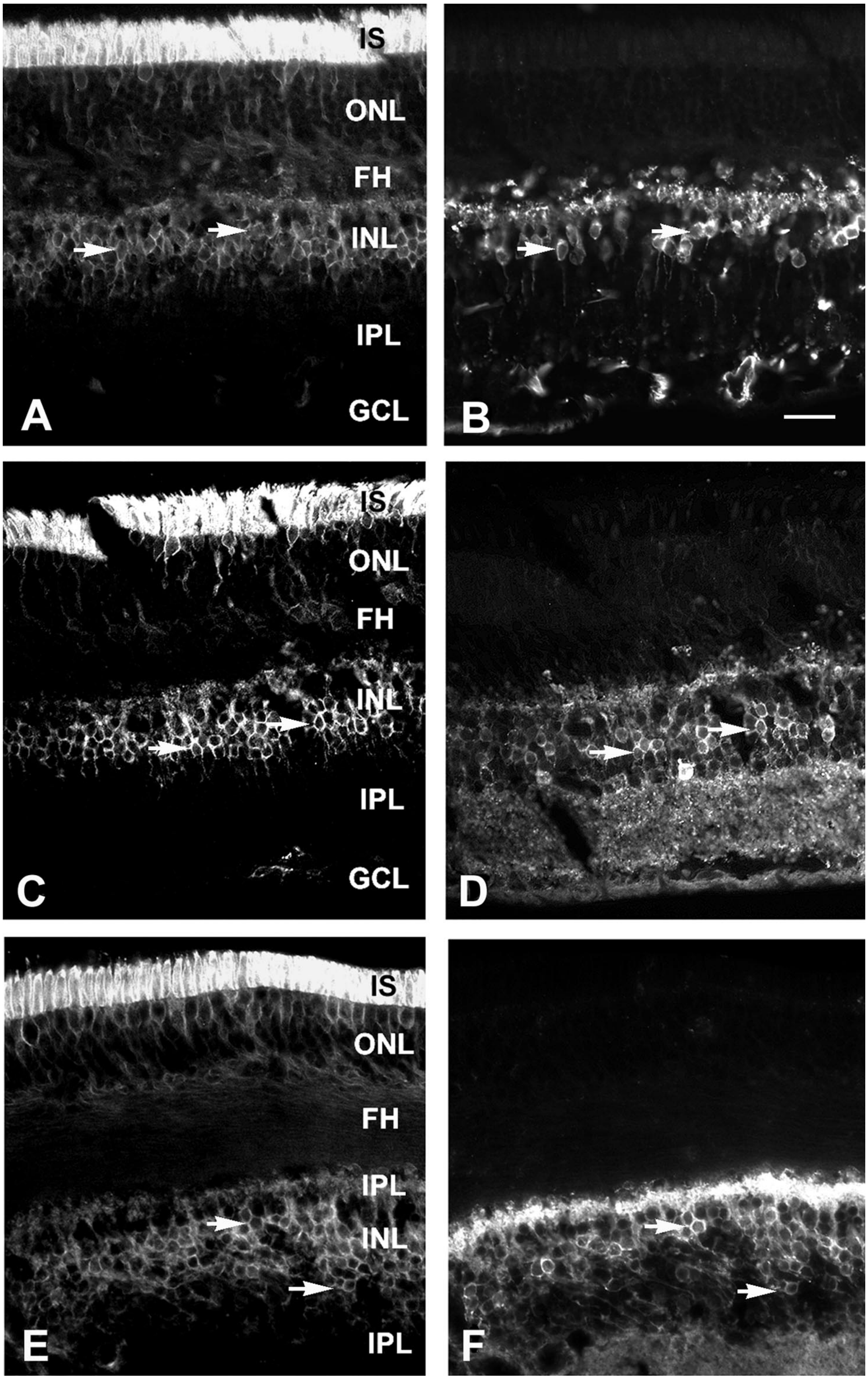

FIGURE 5. Immunofluorescence microscopy of human macular sections double labeled with RS1 antibody and antibodies to bipolar cells. (**A**) RS1 labeling and (**B**) PKCα labeling of rod bipolar cells; (**C**) RS1 labeling and (**D**) PKCβ labeling of cone off-bipolar cells; (**E**) RS1 labeling and (**F**) ROB antibody labeling of a subset of cone on- and rod bipolar cells. *Arrows*: Examples of double-labeled cells. Abbreviations defined in Figure 3. Bar, 20 μm.

the inner segments of foveal and macular cone photoreceptors (Figs. 4A, 4C). The distal tips of cone inner segments were intensely labeled. There was also moderate staining of photoreceptor cell bodies and a few inner retinal cells, presumably bipolar cells (discussed later) that were distributed close to the inner limiting membrane. Double labeling of foveal sections with Müller-cell–specific vimentin antibody,[27] clearly demonstrated that RS1 immunostaining was distinct from Müller cell staining (Figs. 4A, 4B). In the macula, the pattern of RS1 staining was similar to that observed for peripheral retina (Fig. 4C): Very prominent labeling of rod and cone inner segments, strong labeling of cone cell bodies, and moderate labeling of Henle fibers and many cells within the INL. RS1 labeling was absent from the INL margins and entire GCL, and distinct from the vimentin and CRABLP labeling of Müller cells (Fig. 4D).

To more precisely identify inner retinal cells that were immunopositive for RS1, human macula sections were double labeled with RS1 and antibody markers for bipolar cell sub-

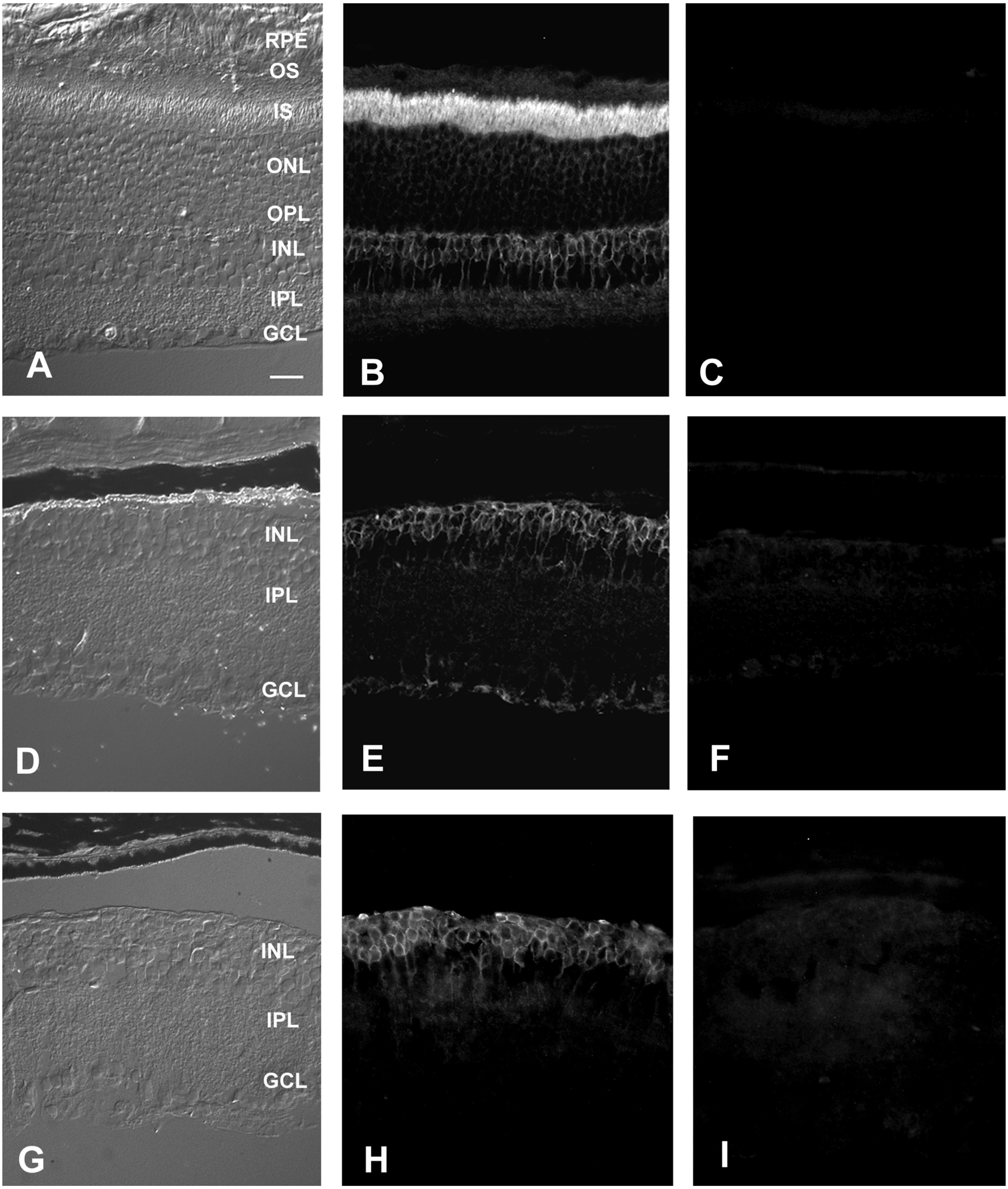

FIGURE 6. Immunofluorescence microscopy of retina from 2-month-old, wild-type and photoreceptorless mouse retina. Wild-type mouse retina: (**A**) Differential interference contrast (DIC) image; (**B**) RS1 immunolabeling; (**C**) Control, RS1 immunolabeling in the presence of excess competing RS1 peptide. *Rd/rd* mouse retina without rod photoreceptors and most cone cells: (**D**) DIC image; (**E**) RS1 immunolabeling; (**F**) control, RS1 immunolabeling in the presence of excess competing RS1 peptide. Similar results were obtained in a 12-month-old mouse. *Rd/rd* cl mouse retina without all rod and cone photoreceptor cells. (**G**) DIC image; (**H**) RS1 immunolabeling; (**I**) control, RS1 immunolabeling in the presence of excess competing RS1 peptide. Abbreviations defined in Figure 3. Bar, 20 μm.

classes.[26] These included PKCα specific for rod bipolar cells, PKCβ specific for cone off-bipolar cells and ROB MAb 115A10 specific for a subclass of cone on-bipolar cells and rod bipolar cells.[22,26] Figure 5 shows that RS1 immunoreactivity was associated with the majority of bipolar cell types labeled with PKC and ROB antibodies (staining with RS1 and a cocktail of the three mAbs produced coincident labeling in >90% of the cases, data not shown).

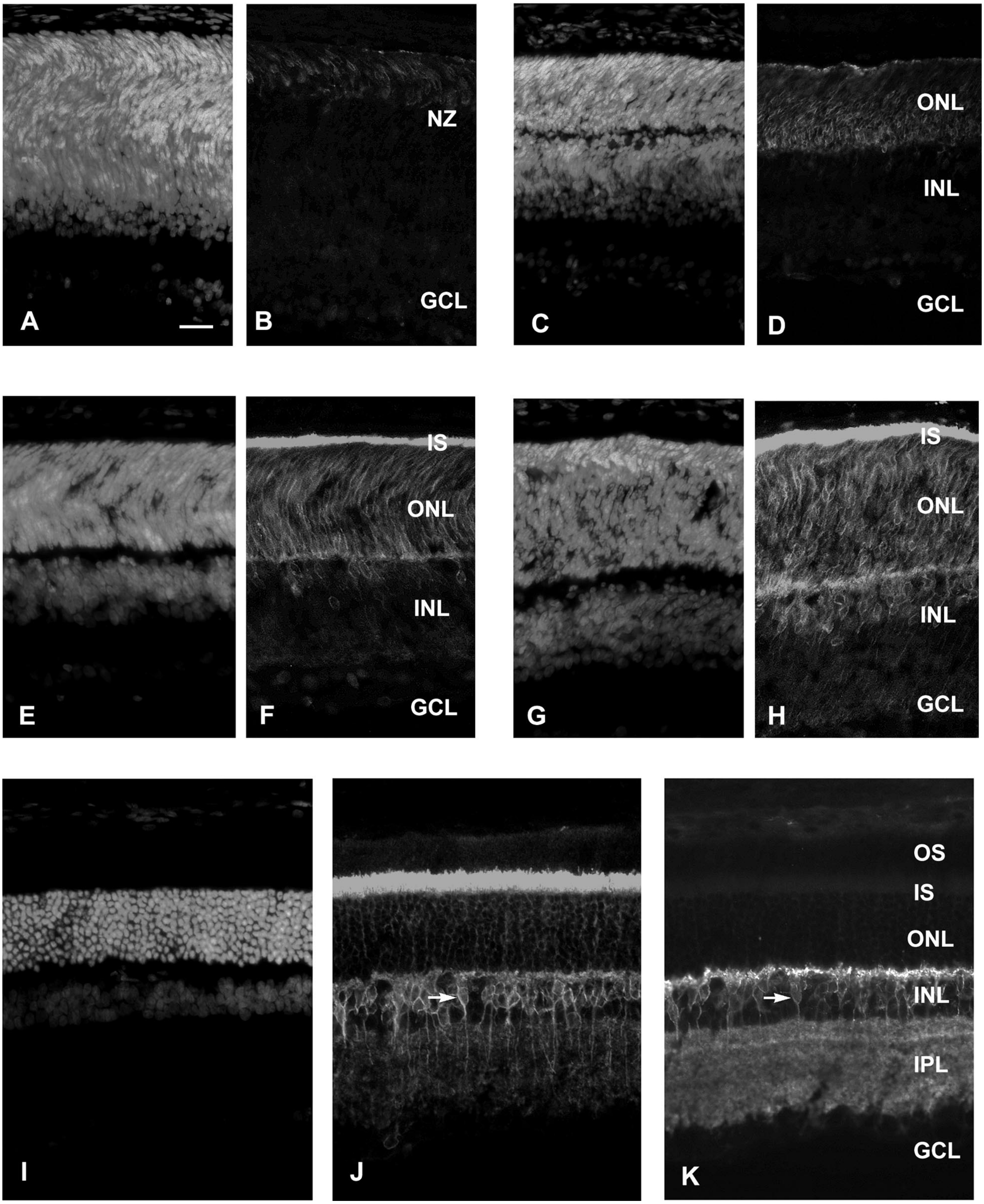

Figure 7. Immunofluorescence microscopy of developing rat retina. Retina from a 6-day-old rat stained with (**A**) DAPI and (**B**) RS1 antibody; retina from an 8-day-old rat stained with (**C**) DAPI and (**D**) RS1 antibody; retina from a 10-day-old rat stained with (**E**) DAPI and (**F**) RS1 antibody; retina from a 12-day-old rat stained with (**G**) DAPI and (**H**) RS1 antibody; retina from adult rat stained with (**I**) DAPI, (**J**) RS1 antibody and (**K**) ROB antibody. *Arrows*: Example of a bipolar cell labeled with both the RS1 and ROB antibodies. Bar, 20 μm.

RS1 Staining of Photoreceptorless Mice

The retina of wild-type and *rd/rd* mice were labeled with the RS1 antibody to determine whether inner retina cell labeling persisted in the absence of a photoreceptor cell layer (Figs. 6A through 6F). As in the case of wild-type retina, the RS1 antibody specifically stained the cell bodies and processes of bipolar cells in *rd/rd* mice 2 and 12 months old. Irregular immu-

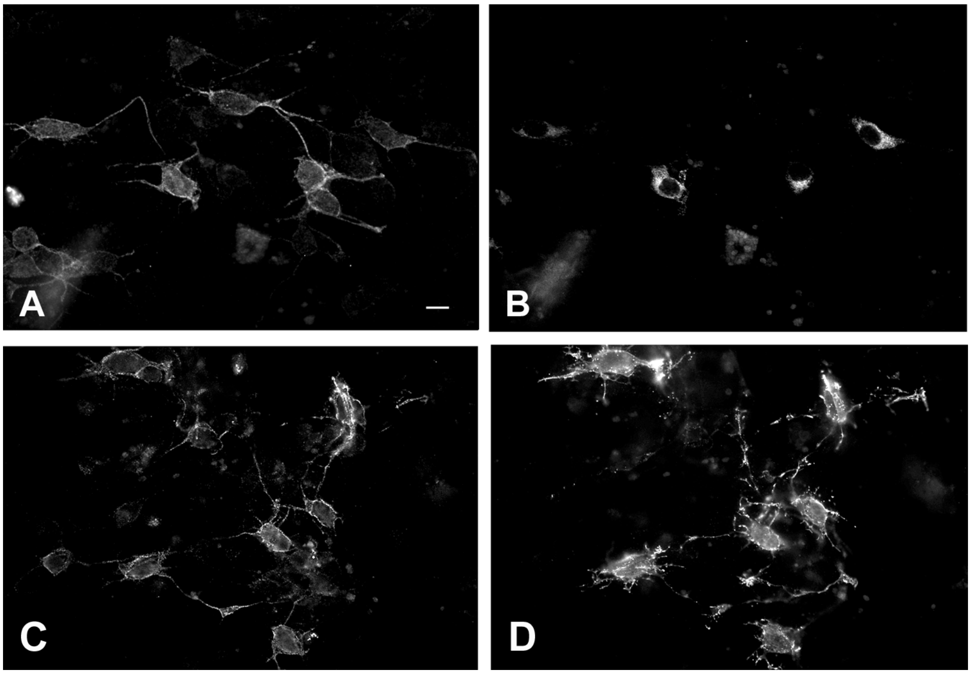

FIGURE 8. Immunofluorescence microscopy of 4-week-old retinal cell cultures double labeled with the RS1 antibody and either PKCα antibody (a marker for rod bipolar cell) or MAb 115A10 antibody (a marker for subclasses of cone and rod bipolar cells). (**A**) RS1 labeling and (**B**) PKCα labeling of the same culture. Some of the RS1-positive cells were also PKCα positive, although the distribution of label was different. (**C**) RS1 labeling and (**D**) MAb 115A10 labeling of the same culture. Most, but not all, RS1-positive cells are also MAb 115A10–positive. Bar, 10 μm.

nostaining below the GCL was observed in *rd/rd* mice that was not observed in wild-type mice. Because *rd/rd* mice are known to contain a small number of surviving cone photoreceptors, we labeled the retina of an *rd/rd* cl mouse, which is without both types of photoreceptors.[28] As shown in Figure 6H, the RS1 antibody specifically stained bipolar cells of the inner retina similar to that observed for wild-type and *rd/rd* mouse.

Developmental Expression of RS1

The temporal expression and distribution of RS1 during development of the rat retina was also examined by immunofluorescence microscopy (Fig. 7). At P6, weak RS1 staining was seen along the scleral margin of the neuroblastic zone, and by P8, labeling of the newly forming photoreceptor outer nuclear layer was evident. The intensity of outer retinal staining increased over time with the emergence of intense staining of the newly formed inner segment layer at P10. Weak RS1 labeling of inner retinal cells just below the photoreceptor cell layer was also first observed at this time. By P12, the adult pattern of expression was present. Staining continued to intensify with age, and as in the case of human retina, many RS1-immunopositive cells in the adult rat INL were also stained with the bipolar-specific ROB MAb 115A10 antibody (Figs. 7J, 7K).

Expression of RS1 in Cultured Retinal Bipolar Cells

RS1 expression in retinal cell cultures derived from enzymatically dissociated retinal tissue was examined by immunofluorescence microscopy. No RS1 immunolabeling was detected for retinal cells maintained in culture for 1 week (data not shown). However, after 4 weeks, a significant number of cells were labeled with the RS1 antibody (Fig. 8A). Diffuse labeling extended to the periphery of the cells, a pattern characteristic of cell surface labeling of nonpermeabilized cells.[21] A number of RS1 positive cells were also PKCα positive indicating that they were rod bipolar cells (Fig. 8B). PKC labeling, however, was primarily localized around the nucleus, a pattern characteristic of intracellular labeling. A large number of RS1-positive cells also labeled with MAb 115A10 indicating that RS1 was expressed in a subset of cultured on-bipolar and rod bipolar cells. Horizontal and ganglion cells in these cultures were not labeled with the RS1 antibody, as revealed in double-labeling studies using cell-specific antibodies.[23]

DISCUSSION

Recently, *Rs1h* mRNA expression has been detected in photoreceptors, but not in other retinal cells by in situ hybridization and Northern blot analysis of wild-type and *rd/rd* mouse retinal RNA.[14] These investigators also reported that an anti-RS1 antibody labeled not only photoreceptor cells, but also Müller cells,[29] ganglion cells, and other unidentified cells of the inner retina.[30] On the basis of these results, they concluded that RS1 is synthesized only in photoreceptors for export into the extraretinal space. It then migrates into the inner retina where it associates with the various cells and structures.

In the present study, we have confirmed that RS1 is abundantly expressed in rod and cone photoreceptor cells. However, our results differ from the earlier studies cited in two important respects. First, our studies indicate that RS1 is expressed in bipolar cells of the inner retina as well as photoreceptors, although at lower levels. Second, double-labeling experiments using a variety of cell surface markers reveal that RS1 is specifically associated with rod and cone photoreceptors of the outer retina and bipolar cells of the inner retina, but not Müller cells, ganglion cells, or the inner limiting membrane.

Several lines of evidence indicate that RS1 is expressed in retinal bipolar cells. By immunofluorescence microscopy, we detected RS1 on bipolar cells of all mammalian retinas examined including 1-year-old *rd/rd* mice without rods and most cones, and *rd/rd* cl mice devoid of both rod and cone photoreceptors. We also observed RS1 expression on bipolar cells dissociated from retinal tissue and maintained in culture for 4 weeks. Because RS1 was not detected in these cells after 1 week in culture, we conclude that RS1 expression in bipolar cells maintained in culture for 4 weeks arises from newly synthesized protein. Finally, although we did not detect *Rs1h* mRNA expression in *rd/rd* mouse retina by Northern blot analysis, in agreement with the results of Reid et al.,[14] we detected *Rs1* expression using the more sensitive technique of RT-PCR. Together, these results provide strong evidence that RS1 is expressed in bipolar cells as well as in photoreceptors.

It is becoming increasingly clear that in situ hybridization and Northern blot analysis are useful techniques to detect abundantly expressed mRNA but often do not have the sensitivity or reliability to measure low-level mRNA expression. For example, the transcription factor *Crx* was initially thought to be expressed only in photoreceptors of the retina on the basis of a strong in situ hybridization signal in the photoreceptor layer and no signal[18,31] or an extremely weak signal[32] in the inner retina. In the present study, we also were unable to detect *Crx* expression in *rd/rd* mice by Northern blot analysis. However, recent immunocytochemical labeling studies have established that *Crx* is also expressed at low levels in retinal bipolar cells.[33] The photoreceptor ABC transporter *ABCA4* (formerly *ABCR*) mRNA expression has been observed in rod, but not cone, cells by in situ hybridization.[34] Recently, however, *ABCA4* has been detected in human cone as well as rod cells by immunofluorescence microscopy and Western blot analysis techniques, a result that has important implications in the pathogenesis of Stargardt disease.[35] These and other studies indicate that caution must be exercised in interpreting cell-specific expression when relatively insensitive techniques are used. The inability to detect RS1 expression in nonphotoreceptor cells by conventional in situ hybridization and Northern blot analysis is most likely due to the limited sensitivity of these techniques.

Immunofluorescence labeling studies using highly specific RS1 antibodies and a variety of cell-specific markers identified the RS1-immunopositive inner retinal neurons as rod and cone bipolar cells in all mammalian species examined. RS1 labeling was not detected on Müller glial cells, ganglion cells, or along the inner limiting membrane of either peripheral or central retina. These results are in marked contrast to previous reports showing more widespread retinal tissue labeling.[29,30] It is unclear why the antibodies used in our study showed selective staining of photoreceptors and bipolar cells, whereas an N-terminal antibody used by another group of investigators[29,30] labeled photoreceptors and most other cells of the inner retina. However, it is interesting to note that the antibody used in later studies labeled multiple bands in Western blot analysis of retinal extracts, leading one to question the specificity of this antibody.

The *RS1* gene codes for a polypeptide that has primary structural features characteristic of an extracellular cell adhesion protein.[9] It has an N-terminal 23-amino-acid hydrophobic signal sequence with a signal peptidase cleavage site, a signature characteristic of proteins destined for secretion from cells. It also contains a discoidin domain found in discoidin I and II, hemocytin, neuropilin, and other proteins implicated in cell adhesion processes.[9–14,36,37]

Biochemical and immunocytochemical experiments performed in this study support the view that RS1 is a secreted protein complex that interacts with cell surfaces. RS1 from retinal and COS-1 cell membranes was found to migrate as a single 24-kDa polypeptide under reducing conditions and a large (>95-kDa) complex under nonreducing conditions. This indicates that RS1 subunits assemble into a large, disulfide-linked complex within the oxidizing environment of the endoplasmic reticulum lumen. The intense peripheral RS1 staining of bipolar cells and cone inner segments and RS1 labeling of nonpermeabilized cultured bipolar cells is consistent with the localization of a significant fraction of RS1 on the surface of these cells. Together, these results support the model in which RS1 is synthesized in photoreceptor and bipolar cells as a disulfide-linked oligomeric complex. This secreted complex associates with the surface of photoreceptor and bipolar cells. The molecular composition of the RS1 complex and identity of the interacting cell surface components are currently under investigation.

Many missense mutations associated with X-linked juvenile retinoschisis involve the introduction or substitution of cysteine residues.[9,15] It is likely that these cysteine mutations cause misfolding and defective subunit assembly of RS1, as recently reported for the photoreceptor-specific protein, peripherin/rds.[38]

The role of RS1 in the pathogenesis of X-linked juvenile retinoschisis remains to be determined at a molecular level. Earlier histopathologic and electrophysiological studies led to the conclusion that defective Müller cells were directly responsible for the disease state.[5,8] However, expression and localization of RS1 on photoreceptors and bipolar cells suggest that these cells are directly involved in the pathogenesis of X-linked juvenile retinoschisis. The discoidin-like domain of RS1 probably participates in cellular adhesion, possibly by stabilizing the association of the extracellular matrix to the surface of photoreceptors and bipolar cells. Mutations in RS1 that compromise the putative role of RS1 as an adhesion protein could destabilize the retinal tissue. The foveal region may be especially compromised because of its unique cellular organization, constituting a weak spot. This fragility is seen in another human retinal disorder, that of the macular hole in which vitreal shrinkage pulling at the retina leads to detachment at the level of the macula.[39] It is unclear why microcysts form between the GCL and the inner limiting membrane, a region that appears to contain little, if any, RS1. Perhaps, the extracellular matrix required to maintain the integrity of the retinal tissue between the GCL and the inner limiting membrane extends into the INL where it is anchored to retinal bipolar cell surfaces through the RS1 complex. Loss of the RS1 adhesion function in persons with X-linked retinoschisis could lead to destabilization of the matrix and splitting of the inner retina cell layer.

Most persons with X-linked retinoschisis produce ERGs with normal a-waves originating from photoreceptors but reduced or absent b-waves.[3,4] Although the b-wave was initially thought to be derived from Müller cell depolarization, there is growing evidence to suggest that the b-wave originates directly from depolarizing bipolar cells.[3,40,41] Immunostaining showing that RS1 is associated with bipolar cells is consistent with the direct involvement of bipolar cells in b-wave activity and in X-linked juvenile retinoschisis.

In summary, the results of this study indicate that RS1 is expressed in both photoreceptors and bipolar cells as a secreted, disulfide-linked oligomeric protein. On export to the extraretinal space this complex binds to the surface of photoreceptors and bipolar cells where it may function as a cellular adhesion protein to maintain the integrity of the retina tissue.

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