A Retinal Neuronal Developmental Wave of Retinoschisin Expression Begins in Ganglion Cells during Layer Formation

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PURPOSE. Mutations in the retinoschisin gene, RS-1, cause juvenile X-linked retinoschisis (XLR), a dystrophy characterized by delamination of the inner retinal layers, leading to visual impairment. Although the retinoschisin protein (RS) is expressed most abundantly in photoreceptors in the outer retina, XLR disease affects the innermost retinal layers, including the nerve fiber layer that contains retinal ganglion cells (RGCs). Considering the histopathological and electrophysiological characteristics of the clinical disease, the present study was conducted to evaluate the local cellular expression of RS-1 during retinal development.

METHODS. RS protein and RS-1 mRNA were localized to specific retinal cell types in embryonic to adult mice by immunohistochemistry, confocal immunofluorescence microscopy, catalyzed reporter deposition in situ hybridization, and laser capture microdissection/RT-PCR.

RESULTS. RS-1 mRNA was expressed first in RGCs by postnatal day (P)1, after terminal differentiation. Expression then moved posteriorly through the retina in a spatial and temporal developmental wave, as additional neuronal classes were born and synaptic layers were formed. RS was expressed by bipolar cells at a time when these neurons were establishing functional synapses with photoreceptors, evidenced by the appearance of the electoretinogram b-wave between P12 and P14.

CONCLUSIONS. All major classes of adult retinal neurons, with the possible exception of horizontal cells, express RS protein and mRNA, strongly suggesting that retinoschisin in the inner retina is synthesized locally rather than being transported, as earlier proposed, from distal retinal photoreceptors. Continued expression of RS by mature inner-retinal neurons supports the possibility of a therapeutic strategy of protein replacement to treat both infants and adults with XLRs. (Invest Ophthalmol Vis Sci. 2004;45:3302–3312) DOI:10.1167/iovs.04-0156

The thin, multilayered sandwich of retinal neural layers is susceptible to structural failures, as occurs in full-thickness retinal detachment from head trauma1 or from genetic defects, such as collagen gene mutations in Stickler syndrome.2 Another type of retinal structural failure occurs in juvenile X-linked retinoschisis (XLRs), a genetic dystrophy in which the organized retinal layers delaminate.3,4 Clinical signs of retinal structural impairment typically involve splitting of the surface layer from the underlying retina, resulting in wispy, filamentous, neural sheets that float in the vitreous cavity, described in the literature as "vitreous veils."5 This structural delamination has obvious effects on neural signaling and causes loss of sight in the involved regions.6 This X-linked trait affects the vision of men only, whereas haploinsufficiency in female carriers rarely affects vision.1,7 Diagnosis of XLRs in infants suggests an abnormality of early retinal development.8 Severe cases involve full-thickness retinal detachment, for which reattachment surgery is rarely successful,9,10 and total vision loss can ensue in severely involved eyes. This is unlike retinal detachments that occur in normal eyes, which can be treated surgically with great success.

After identification of the retinoschisin gene (RS-1) in 1997,11 several studies examined the cellular localization of this retina-specific protein.12–15 Although the highest expression of retinoschisin protein (RS) is observed in photoreceptors, the histopathological and electrophysiological characteristics of the clinical disease are not consistent with photoreceptor-specific degeneration. Instead, the disease affects the innermost retinal layers containing retinal ganglion cells (RGCs) and their processes.1,16 Electrophysiological data from patients with XLRs demonstrate that whereas the b-wave of the ERG, arising from the bipolar cells, is severely affected, the photoreceptor a-wave remains relatively unaffected.17,18

The apparent action of RS at a distance from the photoreceptors led to proposals that RS protein is secreted by the photoreceptors and transported to the inner retina by Müller glial cells.13,14 Unlike others, we preliminarily reported immunohistochemical evidence that RS is in or surrounds RGCs at the retinal surface (Hiriyanna KT, et al. Invest Ophthalmol Vis Sci. 2004;45:3302–3312) DOI:10.1167/iovs.04-0156

Abstract 3481), which suggests local synthesis of the protein immediately after neuronal birth and terminal differentiation, providing a basis to consider the cell-specific gene expression of RS, a discoidin domain family member,19,20 and its impact on synaptic integrity during retinal layer formation. Originally described as lectins from Dictyostelium discoideum, the discoidin-domain–secreted and transmembrane proteins have been implicated in cell adhesion and cell surface-mediated events. These include guidance of axonal processes and binding to components of the extracellular matrix, an RS-proposed function that could strengthen the adhesion of the inner and outer retinal layers.21

The structural separation of retinal layers and impairment of photoreceptor synaptic function, both presumably occurring during development, provide a basis to consider the cell-specific gene expression of RS, a discoidin domain family member,19,20 and its impact on synaptic integrity during retinal layer formation. Originally described as lectins from Dictyostelium discoideum, the discoidin-domain–secreted and transmembrane proteins have been implicated in cell adhesion and cell surface-mediated events. These include guidance of axonal processes and binding to components of the extracellular matrix, an RS-proposed function that could strengthen the adhesion of the inner and outer retinal layers.21

We have now observed a developmental wave of RS expression immediately after neuronal birth and terminal differentiation. RS expression begins first in RGCs at the retinal surface and then progresses distally through the retina as additional classes of neurons appear, all before most photoreceptors are born. Once all classes of neurons are born and with the ap-
appearant of RS in the outer plexiform layer (OPL) synaptic zone, synaptic function is complete, as is evidenced by the appearance of the electroretinogram b-wave. This study shows that RS is expressed locally by most retinal neurons, arguing against a need for transport from the photoreceptors. RS expression continues in adult retinal neurons, which provides a basis for considering protein replacement in the postnatal retina as a therapeutic strategy for the blinding eye disease XLR.

Materials and Methods

Animals and Sample Preparation

Experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with protocols approved by the National Eye Institute Animal Care and Use Committee. Normal C57BL/6J mice, purchased from Charles River Laboratory (Wilmington, MA), were housed under fluorescent lights on a 12-hour light–dark cycle. Studies were performed on mice aged embryonic day (E)16.5 through postnatal day (P)42 (adult).

Mice were killed with a pentobarbital sodium overdose (300 mg/ kg, intraperitoneally; IP), and the eyes were removed. For immunoblotting and RT-PCR, retinas were dissected, and the protein or RNA was extracted. For immunohistochemistry and in situ hybridization, retinal sections were exposed to heat antigen-unmasking conditions for RS, retinal sections were exposed to 4-μm-thick sections, deparaffinized in xylene, rehydrated in a graded series of ethanol, and rinsed in PBS. To determine the optimum antigen-unmasking conditions for RS, retinal sections were exposed to different concentrations of proteinase K (Sigma) at room temperature for 10 minutes. For other antibodies, sections were exposed to heat (95°C) for 15 minutes in 0.01 M citrate buffer (pH 7.0). All sections were quenched with 0.3% hydrogen peroxide in methanol for 30 minutes, blocked with appropriate normal serum in PBS, and incubated in a humidified chamber at 4°C overnight with specific primary antibodies. Control slides were incubated without antibody or with antibodies preabsorbed with 10 μg of antigen peptide. After overnight incubation, sections were washed three times with PBS, incubated for 1 hour with biotin-conjugated secondary antibody, washed again, and then incubated for 1 hour with the avidin-biotinylated enzyme complex (ABC; Vector Laboratories, Burlingame, CA). Last, the slides were rinsed in PBS, and the antibody complexes were visualized with 3,3′-diaminobenzidine (DAB).

Detection of RS in P42 mouse retinal sections was enhanced by epitope unmasking with proteinase K (Fig. 2). Subsequently higher concentrations of proteinase K revealed additional areas of neuronal cell RS immunostaining.

Confocal Immunofluorescence Microscopy

Flatmounted retinas and 100-μm-thick, agarose-embedded retinal sections were preincubated in a solution containing 5% normal serum, 5% amino acid residues 24–37 of mouse RS (Invitrogen Custom Service, Carlsbad, CA). This same epitope was used by Grayson et al., to produce a similar polyclonal antibody. The antibody was affinity purified (Invitrogen), and the specificity of the antibody was tested by immunoblot analysis of mouse retinal proteins. To prepare the immunobLOTS, retinal proteins were extracted with lysis buffer (Mammalian Cell Lysis Kit; Sigma-Aldrich), and the protein concentration was determined with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Proteins were diluted in sample buffer (LDS NuPage; Invitrogen) containing 50 mM dithiothreitol (Invitrogen) as a reducing agent, separated by electrophoresis on 12% Bis-Tris gels (Invitrogen), and then electrophotitically transferred onto nitrocellulose membranes. RS was detected by chemiluminescence (SuperSignal West Dura Substrate; Pierce Biotechnology). Analysis showed the expected 24-kDa band corresponding to RS and a 48-kDa band thought to be the RS dimer (Fig. 1A, lane 1), also seen by Grayson et al., No immunostained protein was detected when the antibody was preabsorbed with RS peptide (Fig. 1A, lane 2).

Other primary antibodies used in the study were: Pax6 (a gift from Atsushi Kawakami, University of Iowa, Iowa City, IA); Chx10 (Exal Biologicals, Boston, MA); Bn-3b, PKCa, PKCβ, G(o)α, and syntaxin 1 (Santa Cruz Biotechnology, Santa Cruz, CA); glutamine synthetase (GS) (BD Biosciences-Pharmaningen, San Diego, CA); syntrophin and calbindin (Sigma); and postsynaptic density protein95 (PSD95; Upstate Biotechnology, Lake Placid, NY).

Immunohistochemistry

Immunohistochemical analysis of mouse retinal specimens was performed as described previously. Paraffin-embedded eyes were cut into 4-μm-thick sections, deparaffinized in xylene, rehydrated in a graded series of ethanol, and rinsed in PBS. To determine the optimum antigen-unmasking conditions for RS, retinal sections were exposed to different concentrations of proteinase K (Sigma) at room temperature for 10 minutes. For other antibodies, sections were exposed to heat (95°C) for 15 minutes in 0.01 M citrate buffer (pH 7.0). All sections were quenched with 0.3% hydrogen peroxide in methanol for 30 minutes, blocked with appropriate normal serum in PBS, and incubated in a humidified chamber at 4°C overnight with specific primary antibodies. Control slides were incubated without antibody or with antibodies preabsorbed with 10 μg of antigen peptide. After overnight incubation, sections were washed three times with PBS, incubated for 1 hour with biotin-conjugated secondary antibody, washed again, and then incubated for 1 hour with the avidin-biotinylated enzyme complex (ABC; Vector Laboratories, Burlingame, CA). Last, the slides were rinsed in PBS, and the antibody complexes were visualized with 3,3′-diaminobenzidine (DAB).

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bovine serum albumin (BSA), 0.1% saponin, and 0.05% sodium azide in PBS at 4°C for 4 hours, followed by incubation with the RS antibody combined with another primary antibody (double labeling) at 4°C overnight. Sections were rinsed with PBS and incubated for 2 hours with secondary antibodies (Alexa Fluor 555 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse; Molecular Probes, Eugene, OR) and with DAPI (Molecular Probes). Samples were washed again and coverslipped in gel mounting medium (Gem Mount; Biomeda, Foster City, CA) as an antifading agent. Control slides were processed similarly, except for the omission of primary antibodies. Slides were examined with a confocal microscope (TCS SP-2; Leica Microsystems, Exton, PA).

**RNA Isolation and RT-PCR**

Total retina RNA was extracted (RNeasy; Qiagen, Valencia, CA), and first-strand synthesis was performed for 50 minutes at 42°C with 1 μg total RNA and reverse transcriptase (Super Script II; Invitrogen). Reactions without reverse transcriptase were used as the negative control. DNA products were amplified by PCR. Initial denaturation was for 12 minutes at 95°C, followed by 35 PCR cycles (denaturation for 30 seconds at 95°C, annealing for 45 seconds at 56°C, extension for 1 minute at 72°C), with a final extension for 10 minutes at 72°C. Primers for PCR amplification were RS-1 5′-TGGGATTTTGAATCACTGAA-3′ (forward) and 5′-GGCTGAGTGAAGTCTCAA-3′ (reverse); and β-actin 5′-TCATGAAGTGGTGTGTTTCA-3′ (forward) and 5′-CTTATAAGGAAGAGGAAC-3′ (reverse). PCR products were separated electrophoretically on 1.2% agarose gels and visualized by ethidium bromide staining. RT-PCR products were con-

**Digoxigenin-Labeled cRNA Probe**

The RS-1 cRNA probe was prepared following manufacturer's protocol (TOPO Tools; Invitrogen). RS-1 primers were designed to contain the RS-1 sequence used in Figure 1B combined with an 11-nucleotide topoisomerase 1 recognition sequence (italic) 5′-CGGACAGGGCTGTTGAGTATCACGACA-3′ (forward) and 5′-TGAAGTCAGGAAGCCCTGATGTTTCAA-3′ (reverse). PCR-generated products were verified by sequencing to contain the topoisomerase site and were subsequently joined (TOPO Joining; Invitrogen) to the T′ 5′ element (sense) and the T3′ element (antisense). A second PCR was performed with the joined RNA template and specific T7′ and T3 primers promoters 5′-GATGACTCGTAATACGACTCACTAT-3′ (forward) and 5′-GAGCCTGCAAAGGCGCTGTA-3′ (reverse). PCR-generated products were verified by sequencing to contain the topoisomerase site and then were subsequently joined (TOPO Joining; Invitrogen) to the T′ 5′ 5′ element (sense) and the T3′ element (antisense). A second PCR was performed with the joined RNA template and specific T7′ and T3 primers promoters 5′-GATGACTCGTAATACGACTCACTAT-3′ (forward) and 5′-GAGCCTGCAAAGGCGCTGTA-3′ (reverse). Conditions for both PCR reactions were the same: initial denaturation for 12 minutes at 95°C; 35 PCR cycles (denaturation for 30 seconds at 95°C, annealing for 45 seconds at 60°C, and extension for 1 minute at 72°C) and a final extension for 10 minutes at 72°C. The first and second PCR products were purified and checked for accuracy by direct sequencing. The second PCR template was used for in vitro transcription of sense and antisense digoxigenin (DIG)-labeled RS-1 RNA probes (DIG RNA Labeling Kit; Roche Diagnostics, Indianapolis, IN). The reaction product was confirmed by gel electrophoresis.

**Northern Blot Analysis**

Northern hybridization was performed to verify the specificity of the DIG-labeled RS-1 cRNA probe. Mouse total RNA (10 μg) and RNA molecular weight markers (New England BioLab, Beverly, MA) were electrophoresed on a formaldehyde-1% agarose gel and transferred onto a positively charged nylon membrane (Roche Diagnostics). The membrane was photo cross-linked at 120 μJ (Stratalinker; Stratogene, La Jolla, CA). Prehybridization and hybridization were then performed (DIG Easy Hyb; Roche). The blot was hybridized with the DIG-labeled antisense RS-1 RNA probe at 42°C for 16 hours. Membranes were washed twice for 5 minutes in 2× SSC/0.1% SDS at room temperature and then twice for 15 minutes at 68°C in the same buffer. A final stringent wash was performed for 25 minutes in 0.1× SSC/0.1% SDS at 68°C. Hybridization of the mRNA was detected by chemiluminescence (CDPStar kit; Roche), and the specificity of the RS-1 cRNA probe was confirmed (Fig. 1C). The probe detected the expected 5.5 kb RS-1 mRNA band. No extra bands were observed.

**Catalyzed Reporter Deposition In Situ Hybridization**

Catalyzed reporter deposition (CARD), a highly sensitive technique, was used to localize RS-1 mRNA in the retina. Compared with conventional techniques, CARD with tyramide signal amplification significantly enhances the chromogen reaction with substantial gain in morphologic resolution.24 In preparation, paraform-embedded, 4-μm-thick retinal sections were deparaffinized and rehydrated in a graded series of ethanol, incubated in 20 μg/mL proteinase K for 20 minutes, postfixed with 4% PFA/PBS, and immersed for 15 minutes in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic acid. The sections were then washed in PBS and hybridized with the DIG-labeled anti-sense or sense (control) RS-F RNA probe overnight at 42°C in a moist chamber. After hybridization, the slides were washed with solutions containing 50% deionized formamide and decreasing amounts of SSC (2×, 1×, 0.2×, and 0.1×) at 42°C, followed by a final wash in Buffer 1 (0.1 M Tris-HCl [pH 7.5], 0.2% Triton X-100, and 0.5 M NaCl) at room temperature. The slides were then visualized with a signal-amplification system (TS Biotin System; PerkinElmer, Boston, MA). As outlined in the system protocol, the slides were first treated with a 1.5% blocking agent in Buffer 1 for 1 hour, followed by incubation in a 1:100 dilution of horseradish peroxidase (HRP)- conjugated DIG antibody (Roche) for 1 hour at room temperature. Slides were then washed twice with Buffer 1, blocked with blocking buffer (TNB; PerkinElmer), and incubated in a 1:50 dilution of biotinyl tyramide reagent in the dark for 10 minutes at room temperature. After three washes for 5 minutes in Buffer 1, the slides were incubated in streptavidin-HRP in the dark for 1 hour at room temperature. After three more washes for 5 minutes in Buffer 1, the slides were incubated with DAB. After a wash in distilled water to stop the chromogenic reaction, the slides were counterstained with methyl green and mounted for microscopy.

**LCM and RT-PCR**

Methacarn-fixed, 8-μm-thick retinal tissue sections were mounted on uncharged glass slides (Erie Scientific, Portsmouth, NH). The sections were stained (HistoGene solution; Arcturus, Mountain View, CA) for 15 seconds and washed with RNase-free water for 30 seconds. The sections were dehydrated in graded ethanol solutions (75%, 95%, and 100%), cleared in xylene, air dried for 30 minutes, and stored in a desiccator for 2 hours. Samples were placed on LCM film (CaptSure HS Sample Preparation System; Arcturus), and retinal cells were collected (PixCell II LCM system; Arcturus), with parameters set at 7.5 μm diameter laser spot size, 100-mW power, and 0.3 to 1.0-ms duration. The microdissected cells were lysed in buffer (Qiagen), and total RNA was extracted (RNeasy; Qiagen). To eliminate potential genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Invitrogen) for 15 minutes at room temperature. The whole volume of each sample was used as a template for RT-PCR with reverse transcriptase (Super Script II; Invitrogen). Samples were first tested with the β-actin primers 5′-TCACTAAGTTGAGTGAAGTCTCAA-3′ (forward) and 5′-CTTATAAGGAGTGAGTGAAGTCTCAA-3′ (reverse). The same primers spanning exons 5 to 6 that were used to generate the 285-bp product in Figure 1B. Only those samples with efficient amplification of β-actin products were further analyzed with RS-1-specific primers 5′-GTACCCAGAAAACTGACAGAATCTGA-3′ (forward) and 5′-CATCAGGCTGCTGCTTGA-3′ (reverse). This second set of RS-1 primers span exons 3 to 5 and generates a 273 bp RS-1 RT-PCR product, which was confirmed by direct sequencing. Corneal tissue, which does not express RS-1, and reactions without reverse transcriptase were used as negative controls.
Electroretinography

Mice were dark adapted for 12 hours, anesthetized (IP) with ketamine (70 mg/kg) and xylazine (6 mg/kg), and placed under dim red light. Pupils were dilated with 0.1% atropine and 0.1% phenylephrine HCl. Body temperature was maintained near 38°C with a warming pad. A gold wire loop electrode was applied to the cornea after treatment with 1% tetracaine topical corneal anesthesia. A gold wire differential electrode was placed on the sclera near the limbus, and a wire was attached to the ear as the neutral electrode. Responses were amplified at 5000 gain, 0.1 to 1000 Hz. Xenon photostrobe full-field 30-µs flashes had 0.6-log cd · s/m² maximum intensity. Photopic cone ERGs were elicited with 0.6 log cd · s/m² stimuli on a rod-suppressing 42 cd · s/m² white background.

RESULTS

Developmental Retinal Expression of RS Protein

RS protein and RS-1 mRNA expression was restricted to the innermost region of the retina early in development (Figs. 3, 4). The RGCs were the only identifiable cells labeling for RS at E16.5 (data not shown) and P1. Essentially, no other labeling was seen in the undifferentiated neuroblastic layer (NBL), including the region in which photoreceptors subsequently developed. The labeling of RGCs occurred after most of these cells were born and were expressing their signature transcription factors Pax6 and Brn3b, signaling terminal differentiation (Fig. 5). Pax6 is a master regulatory gene essential for early retinal specification. It is spatially restricted to the inner retina during development and localizes to ganglion, amacrine, and horizontal cells in the adult. Brn3b is a transcription factor of the POU homeodomain subclass and is expressed in differentiating RGCs. RS labeling of RGCs continued through adulthood but decreased in intensity (Fig. 3).

Amacrine cell differentiation and Pax6 expression at the inner margins of the NBL began at P1, shortly after the RGCs (Fig. 5), but RS immunolabeling was not apparent until P3, consistent with RS expression after and not preceding neuronal terminal differentiation. RS labeling of amacrine cells tapered in later developmental ages, from P7 onward. By P3, some cells at mid depth of the NBL labeled for RS in a pattern that overlaid Pax6 labeling and was consistent with horizontal cells. By P7, Pax6 labeled horizontal cells discretely at the distal inner nuclear layer (INL) border adjacent to the incipient OPL, but RS antibody no longer localized to these cells. Adult horizontal cells and processes were calbindin positive but did not colocalize RS (Fig. 6), suggesting that horizontal cells may express RS transiently during development, but not in the mature retina.

The OPL became evident by P7, and INL cells, identified with Chx10 as rod bipolar cells, began to label for RS (Figs. 3, 5). Chx10 is a paired homeodomain transcription factor expressed in the retinal neuroepithelium and in rod bipolar cells. RS immunolabeling of photoreceptors was visible by P7 and increased by P10, once a substantial number of inner segments (IS) appeared. The INL narrowed progressively between P7 and P14, because of apoptotic developmental loss of neurons demonstrated in rodent and human, and by P14, RS immunolabeling was evident across essentially the entire width of the INL.

RS-1 mRNA by In Situ Hybridization and LCM

Both in situ hybridization (Fig. 4) and LCM (Fig. 7) demonstrate that the RGCs were the source of the RS at the surface of the retina. The RGCs clearly contained RS-1 mRNA as early as P1. RS-1 mRNA was also detected in the ONL and the INL at P14.

FIGURE 3. RS immunolabeling of mouse retina at ages P1 to P42. RS (left), antigen preadsorption negative controls (right). At P1, labeling was limited to RGCs. Labeling was seen in progressively more posterior regions with time. Labeling of photoreceptors became evident by P7 and more extensively by P10 to P14. Scale bar, 25 µm.
and the distribution of RS-1-positive signal approximated the pattern seen in the adult retina.

Because local expression of RS protein by retinal neurons other than photoreceptors and bipolar cells has not been reported, we confirmed the in situ results by performing RT-PCR on RGCs isolated by LCM (Fig. 7). RS mRNA was readily amplified by RT-PCR from the RGC layer at P1 and also in the adult retina. PCR products for RS-1 and β-actin were detected at the expected molecular sizes of 273 and 285 bp, respectively, and negative controls (samples without reverse transcriptase and corneal epithelium) did not show positive bands. One can conclude that RGCs express RS both developmentally and in the adult retina, indicating that local replenishment of RS protein evidently is desirable for maintaining retinal structure, even after retinal development is completed.

**Synaptic Developmental RS Labeling and Establishment of Function**

The synaptic junction between photoreceptors and second-order retinal neurons lies midway in the OPL, and this zone showed RS labeling by P10 to P14 (Fig. 3). In the adult retina, RS immunolabeling of the photoreceptor synapse appeared as barlike structures. The RS labeling abutted but did not overlay synaptophysin labeling (Fig. 6) or PSD95 (not shown). RS labeling of the synapse between photoreceptors and second-order neurons occurred on the postsynaptic side. Synaptophysin is an integral membrane protein expressed in most retinal synapses on the presynaptic side. PSD95 belongs to a family of ion channel clustering molecules and in the retina labels presynaptically within the photoreceptor terminals. Although RS-1 is expressed to a considerable extent in photoreceptor IS, RS localized to the INL side of both synaptophysin- and PSD95-positive synaptic structures.

ERG recordings showed the first appearance of the b-wave by P14 but not at P12, indicating that synaptic functionality develops between these ages (Fig. 8). The positive-going b-wave of the dark-adapted ERG response is generated postsynaptically to the photoreceptors, whereas the photoreceptors generate a negative-going a-wave that precedes the b-wave in the dark-adapted ERG tracings. The a-wave appears earlier in development than the b-wave, even though both the bipolar and photoreceptors cells develop before P11. The b-wave appearance after P12 is an indication that synaptic function occurred co-temporally with RS-1 expression within the OPL synaptic layer. The emergence of synaptic function also coincides with vesicular glutamate transporter (VGLUT)-1 immunoreactivity predominantly at P12. VGLUT1 serves to load the photoreceptor-bipolar synapse with glutamate neurotransmitter.

**RS Immunoreactivity and RS-1 mRNA in the Adult Retina**

The photoreceptor IS were the most prominent site of RS immunoreactivity in the adult retina. The pattern of RS distribution in the developing mouse retina correlated with terminal differentiation of classes of retinal neurons and with layer formation. Neuronal birth of each neuronal subtype occurs...
over the course of several days. In all cases that we checked, these neurons were immunoreactive for their specific identifying transcription factors before RS appearance, and RS followed neuronal differentiation rather than driving it. Consequently, one would anticipate a role for RS in the mature retina, in addition to that in development. With this in mind, we surveyed the adult retinal neurons for RS immunoreactivity and for RS-I mRNA (Fig. 9).

RS protein localized to the plasma membrane of all retinal neurons, except horizontal cells (Fig. 6). RS labeled all classes of bipolar cells, including rod bipolar cells (identified by PKCα41), cone hyperpolarizing (OFF)-bipolar cells (colabeled with PKCβ42), and cone depolarizing (ON)-bipolar cells (colabeled with G(α)α, which also marks rod bipolars43,44). RS also colocalized to bipolar processes through the INL and to the photoreceptor synaptic complexes with PKCβ and G(α)α. This is consistent with our ERG analysis of XLRS-affected men, which implicated defective signaling through both cone hyper- and depolarizing bipolar cells.17

**Figure 6.** Confocal images of double-labeled retinal bipolar cells from adult (P42) mice, at low and high magnification. RS label in red (left). Middle panels in green labeled for (A) PKCα-specific for rod bipolar cells, (B) PKCβ, cone hyperpolarizing bipolar cells; (C) G(α)α, cone and rod depolarizing bipolar cells; (D) calbindin, horizontal cells and processes; and (E) synaptophysin, presynaptic at the photoreceptor-bipolar synapse. Overlay images show double labeling of all these RS-positive cells and processes (right) with the exception of horizontal cells in (D). The RS labeling in (E) was postsynaptic to synaptophysin. The RS-positive, barlike synaptic structures in the OPL were double labeled with both PKCβ and G(α)α, indicating involvement of both depolarizing and hyperpolarizing bipolar cells. Scale bar, 20 μm.

**Figure 7.** LCM and RT-PCR of the P1 retina and P42 adult mouse retina and cornea. (A) Photographs of LCM showing before, after, and captured specimens of P1 retina (top row) and P42 adult retina and cornea (remaining rows). (B) RT-PCR, with (+) or without (−) reverse transcriptase, of the RS-I 273-bp product (top) and the β-actin 285-bp product (bottom). Ganglion cells at P1 show expression of RS-I. The corneal epithelium serves as an ocular control tissue by the absence of an RS-I RT-PCR product. Samples shown are DNA markers (M) and products from normal retinal lysate (P), negative control of retinal lysate (without reverse transcriptase) (N), ganglion cell layer (GCL), outer nuclear layer (ONL), and corneal epithelium (CE).
Adult retinal amacrine cells also showed colocalization of RS with syntaxin (data not shown), an amacrine marker, in agreement with RS-I mRNA in cells along the lower INL margin by in situ hybridization in Figure 9A. In situ hybridization using CARD/tyramine signaling, a method with enhanced sensitivity compared with conventional techniques, readily demonstrated RS-I mRNA in retinal neuronal cells other than the photoreceptors (Fig. 9A). The RS-I antisense probe gave a strong positive signal in the photoreceptor IS of the adult retina. Detectable signal was also evident in ONL cells, in both the outer and inner regions, consistent with expression by both bipolar cells and amacrine cells. RGCs consistently labeled for RS-I mRNA in situ, corroborating LCM studies showing RS-I mRNA in adult RGCs, along with cells isolated by LCM from the INL (Fig. 7). The results were convincing, as the RS-I sense probe gave no signal.

Müller Glial Cells

Early studies have reported RS expression in photoreceptors and suggest that the protein is transported to the inner retina by Müller cells. Our results, based on RS/GS double-labeling, showed no evidence of RS in Müller cells (Figs. 9B, 9C). GS is a specific marker for Müller cells. This protein did not colocalize with RS in either the perikarya or Müller cell processes. Neither was RS immunolabeling evident at the retinal surface where the Müller cell end feet processes form the inner limiting membrane (ILM) at the vitreous border.

DISCUSSION

Developmental Wave of RS1 Expression from Proximal to Distal Retina

This study demonstrated that essentially all retinal neurons express RS during development. The initial developmental burst of RS expression occurred at the retinal surface by P1, after maturation of RGCs. Expression then occurred progressively in more distal retinal layers as successive classes of neurons terminally differentiated from the undifferentiated NBL. Expression continued in the mature retina in all classes of neurons to a lesser degree, with the apparent exception of adult horizontal cells. The localization of RS to the postsynaptic elements in the OPL correlated with the time that synapses became functional and propagated visual signals, as monitored by the developmental appearance of the ERG b-wave by bipolar cells postsynaptic to the photoreceptors.

Retinal neurons differentiate into different retinal cell types in an orderly fashion and in fixed chronological sequence. RS-I expression roughly followed the same order of appearance. This resulted in a temporal and spatial developmental wave of RS expression across the retinal layers (Fig. 10). Although photoreceptor IS begin to appear by P5, and some even earlier, they do not mature as a full class of cells until later. Previous data indicate that RS-I mRNA and RS protein appear in the retina by approximately P5, thereby coinciding with the earliest (although not mature) development of photoreceptor IS. The present data, however, using protein epitope unmasking and highly sensitive in situ CARD analysis, demonstrated both RS protein and RS-I mRNA in RGCs by P1. Consequently, it seems unlikely that the presence of RS in the proximal retina by P1 is due to production by rods or cones with transport by Müller glial cells, as suggested by other studies. Further mediating against RS transport from distal to proximal retina, Müller cells do not span the retinal thickness by P1.

Müller Cells in XLRs

Despite using extensive epitope unmasking that readily revealed RS protein throughout the retina, we did not see RS immunoreactivity in Müller cells or processes, although this has been reported by others. RS localized with bipolar cell axons that run tightly parallel and immediately adjacent to Müller processes (Fig. 9), which may have led to the prior interpretation of immunohistochemical data. The interpretation of the present data is that RS is secreted by nearly all retina neurons and acts locally, with no evidence of Müller cell expression or transport through Müller cells of RS from outer retinal photoreceptors. However, XLRs disease historically has been attributed to primary pathology in the Müller cells, based in part on the characteristic b-wave absence in ERG responses of affected males. Although the mammalian ERG b-wave was thought to originate from Müller cell activity, recent evidence implicates the bipolar cells directly in b-wave generation. Further, our ERG modeling of human XLRs responses implicates abnormal signaling by both the cone depolarizing and hyperpolarizing bipolar circuits. This feature of the ERG had not been suspected of involving hyperpolarizing bipolar cells. The present data confirm RS involvement in both these cell classes and their synapses. Müller cells may, however, participate secondarily in XLRs. XLRs human histopathology shows strong Müller cell staining for the intermediate filament proteins vimentin and glial fibrillary acidic protein (GFAP) across the retina and in the schisis cavities. Müller cell hypertrophy and vimentin and GFAP expression are nonspecific findings in many retinal diseases. One can surmise that the outer retinal reticular gliotic appearance that is frequently seen clinically in older XLRs-affected males may result from Müller cell hypertrophy. The relatively unaffected Müller cells in the recently engineered RS mouse, Rx1b/Y, confirms that this effect is most likely secondary. Although the heavy expression by photoreceptors has been proposed to couple with Müller glial transport of RS into the proximal retina, there is no compelling rationale for this in the face of local produc-
tion by RGCs and essentially all other developing and adult retinal neurons.

**ERG Synaptic Integrity as a Marker of XLRS Disease Severity**

The degree of b-wave preservation in the clinical ERG of males with XLRS males may provide a useful surrogate for gauging the degree of retinal impairment. The b-wave originates from bipolar cell activity postsynaptic to the photoreceptor synapse in the OPL. Formation of the IPL synapse onto RGCs is already under way at birth\(^{59,60}\) at the time that RS is expressed. OPL ribbon synapses adopt their final morphology between P7 and P12\(^{59,61,62}\) and OPL synapses become functional shortly afterward, as judged by the ERG b-wave appearance between P12 and P14. Only the photoreceptor a-wave is seen before this, at P11 and P12.

We have noted that some XLRS-affected males with clinically mild structural disease retain nearly normal b-wave amplitude,\(^{63}\) and we have further noted in genotyping many XLRS

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**Figure 9.** RS-1 in situ hybridization and double-labeled confocal images of adult (P42) mouse retina. (A) Low magnification of in situ hybridization with RS-1 sense (left) and antisense probes (middle). High magnification with antisense probe (right) in outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Scale bars, 25 μm (low magnification); 10 μm (high magnification). (B) Double labeling for RS (left) and GS (middle) in the retinal Müller glial cells. Right: overlay images. The processes and cell bodies of RS-expressing cells were not colocalized with those of GS-labeled Müller cells. The processes of RS-expressing cells and GS-expressing Müller cells in the IPL paralleled each other. Scale bars: 20 μm. (C) Optical flatmount analysis of RS in the photoreceptor inner segment (IS). GS labeled Müller cells and their microvilli. In the vertical view (top), RS (red) intensity was strong in the IS. Microvilli of Müller cells (green) surrounded the IS but did not colocalize RS. The optical flatmount view (bottom) shows a honeycomb pattern of the photoreceptor IS plasma membranes. GS (green) labeling of Müller cell processes yielded a dotlike pattern that did not colocalize with RS (red).

**Figure 10.** Model of the temporal and spatial wave of RS-1 expression during retinal development. RS expression begins earliest in the RGCs at a time when the class of photoreceptors cells have not differentiated. Expression tapers in the GCL at later times. This is followed at P3 by expression in amacrine cells and only subsequently in photoreceptor cells at a later age. RPC, retinal progenitor cell; GCL, ganglion cell layer.
families that putative protein truncation from RS-1 mutations in exons 1 to 3 roughly correlates with clinically more severe disease, including major ERG b-wave reductions. Because the b-wave originates from bipolar cell activity transsynaptic to the photoreceptors, it reflects the degree of developmental OPL synaptic integrity somewhat directly and may also reflect indirectly the extent of developmental retinal structural abnormality. Hence, the ERG may serve as a tool for predicting the severity of the clinical disease course or to monitor success of therapeutic intervention.

RS Anchoring between Photoreceptors

Although it has not been widely noted in studies of RS protein localization, in addition to delamination and schisis in the inner retinal layers, XLRS clinical disease predisposes to full-thickness retinal detachments that occur between photoreceptors and RPE. The optical en face flatmount retinal view (Fig. 9C) showed heavy RS labeling of the plasma membrane surfaces of photoreceptor IS in a honeycomb pattern. Physiological adhesion mechanisms between photoreceptor outer segments (OS) and RPE have been emphasized through ensheathing of cone and rod OS by RPE apical processes. At the same time, however, this RPE-OS complex must remain plastic, as the OS undergo daily renewal by elongation and shedding. The heavy concentration of RS along the entire IS length could provide considerable lateral anchoring between photoreceptors cell-to-cell to augment structural integrity of the OS region, based on the adhesion function of other discoidin domain proteins. Hence, it is not surprising that surgical repair of XLRS full-thickness detachments are most frequently unsuccessful, as this additional anchoring mechanism would be weakened by RS-1 mutations.

Foveal Radial Cystic Pattern in XLRS

XLRS prototypically involves the human macula and results in formation of elongated, radially oriented foveomacular cysts in a spoked-wheel configuration. The primor fovea is specialized for high spatial resolution and visual acuity provided by high packing density of cone photoreceptors with no rods across the central 350 μm. It is anatomically marked by clinically recognizable retinal thinning that forms the foveal pit due to centrifugal displacement of inner retinal neurons away from the fovea center during later development that occurs after neuronal synaptic connections are already formed. Hendrickson and Kupfer and Hendrickson and Yuodelis have proposed a scheme of primor fovea development involving differential slippage of neuronal layers in two planes to form the foveal pit. Centrifugal displacement of RGCs requires the differential slippage of neuronal layers in two planes to form the foveal pit. Second, the continued expression of RS by adult retinal neurons suggests an ongoing, albeit possibly low, turnover of the RS protein, thereby providing a rationale for intervention even in the developed retina but before catastrophic structural failure occurs to cause a full-thickness detachment. Third, as XLRS disease impairs both synaptic function and retinal structure in development, monitoring synaptic integrity by clinical ERG b-wave recordings may provide a marker to judge disease severity and, conversely, to track successful intervention.

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