Retinoschisin (RS1), the Protein Encoded by the X-linked Retinoschisis Gene, Is Anchored to the Surface of Retinal Photoreceptor and Bipolar Cells through Its Interactions with a Na/K ATPase-SARM1 Complex*

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Retinoschisin or RS1 is a discoidin domain-containing protein encoded by the gene responsible for X-linked retinoschisis (XLRS), an early onset macular degeneration characterized by a splitting of the retina. Retinoschisin, expressed and secreted from photoreceptors and bipolar cells as a homo-octameric complex, associates with the surface of these cells where it serves to maintain the cellular organization of the retina and the photoreceptor-bipolar synaptic structure. To gain insight into the role of retinoschisin in retinal cell adhesion and the pathogenesis of XLRS, we have investigated membrane components in retinal extracts that interact with retinoschisin. Unlike the discoidin domain-containing blood coagulation proteins Factor V and Factor VIII, retinoschisin did not bind to phospholipids or retinal lipids reconstituted into unilamellar vesicles or immobilized on microtiter plates. Instead, co-immunoprecipitation studies together with mass spectrometric-based proteomics and Western blotting showed that retinoschisin is associated with a complex consisting of Na/K ATPase (α3, β2 isoforms) and the sterile alpha and TIR motif-containing protein SARM1. Double labeling studies for immunofluorescence microscopy confirmed the co-localization of retinoschisin with Na/K ATPase and SARM1 in photoreceptors and bipolar cells of retina tissue. We conclude that retinoschisin binds to Na/K ATPase on photoreceptor and bipolar cells. This interaction may be part of a novel SARM1-mediated cell signaling pathway required for the maintenance of retinal cell organization and photoreceptor-bipolar synaptic structure.

Retinoschisin, also known as RS1,3 is a retinal-specific protein encoded by the gene associated with X-linked retinoschisis (XLRS), a leading cause of early onset macular degeneration in males (1). It is primarily expressed in photoreceptors and to a lesser extent bipolar cells of the adult retina (2–4). Retinoschisin consists of a 23-amino acid N-terminal signal peptide and a 157-amino acid discoidin domain flanked by a unique 39-amino acid Rs1 domain and a 5-amino acid C-terminal segment (5, 6). The signal peptide directs the nascent polypeptide across the ER membrane before its removal by a signal peptidase in the lumen of the ER. The processed polypeptide folds into its native conformation and further assembles into a disulfide-linked homo-octameric complex prior to secretion from cells (7). The secreted, disulfide-linked octamer associates with the external surface of rod and cone photoreceptor cells of the outer retina and bipolar cells of the inner retina (3).

The function of retinoschisin is not known at the present time. However, the characteristic features of XLRS patients and retinoschisin knock-out mice suggest that this extracellular protein plays a crucial role in maintaining the cellular organization and synaptic structure of the retina. XLRS is characterized by a splitting of the retinal cell layers, a loss in central vision, and a decrease in the b-wave amplitude of the ERG (8–10). To date over 130 disease-causing mutations in the RS1 gene have been identified. In vitro studies have shown that most disease-causing missense mutations in the discoidin domain of retinoschisin cause protein misfolding and retention in ER of cells (5, 12–14). Mutations in cysteine residues within the Rs1 domain and the C-terminal segment result in normal protein folding and secretion, but defective disulfide-linked octamer formation (5, 7). Retinoschisin knock-out mice exhibit many features found in XLRS pathology (15). These include a marked disorganization of the retinal cell layers with gaps between bipolar cells of the inner retina, disruption of the photoreceptor-bipolar synaptic structure of the outer plexiform layer, reduction in the b-wave amplitude of scotopic and photopic ERGs, and progressive degeneration of rod and cone photoreceptor cells. Together, these studies suggest that loss in retinoschisin function compromises the structural integrity of the retina and disrupts normal signal transmission across the photoreceptor-bipolar synapse.

*This work was supported in part by grants from the National Eye Institute (EY 02422) and the Canadian Institutes of Health Research (MT 5822). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: RS1, retinoschisin; XLRS, X-linked retinoschisis; ER, endoplasmic reticulum; ERGs, electroretinograms; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; FVa, activated Factor Va; GST, glutathione-S-transferase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PB, phosphate buffer.
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The discoidin domain, also known as the F5/8 type C domain, is the main structural unit of retinoschisin (1). Discoidin domains were first discovered in discoidin proteins of the slime mold Dictyostelium discoideum (16). Subsequently, they have been found in a wide variety of extracellular and cell surface membrane proteins that function in cell adhesion, development, and signaling (17). These include blood coagulation Factors V and VIII, milk fat globule protein, neuropilins, neurexin IV, discoidin domain receptor proteins, and others (17, 18).

The high resolution structure of the C2 discoidin domain of Factor V and Factor VIII and the b1 discoidin domain of neuropilin 1 have been determined by x-ray crystallography (19–21). In each case it is composed of a core \( \beta \)-barrel structure in which a 5-strand antiparallel \( \beta \)-sheet packs against a 3-strand antiparallel \( \beta \)-sheet. At one end of the \( \beta \)-barrel, the discoidin domain is stabilized through the formation of a disulfide bond between conserved cysteine residues at the start and end of the discoidin domain. At the opposite end loops or spikes protrude from the core \( \beta \)-barrel structure and define a groove or cleft that serves as the ligand binding site (18, 21).

The structures of the C2 domains of Factor V and Factor VIII have been used to generate a structural model for the discoidin domain of retinoschisin (5, 22). Like Factor V and VIII, it has a \( \beta \)-barrel structure with several loops delimiting a groove.

Discoidin domains have been reported to bind a variety of different cellular components. Blood coagulation proteins Factor V and Factor VIII are known to bind to cell surface anionic phospholipids and in particular phosphatidylserine, whereas discoidin domain receptors I and II specifically interact with collagen and neuropilin 1 binds semaphorin 3, VEGF, heparin and heparin-binding proteins (18, 21, 23). Discoidin I and II of D. discoideum, on the other hand, have been reported to have a strong affinity for carbohydrates and in particular galactose (16).

Although retinoschisin associates with retinal cell surface membranes (3, 24), the identity of the components that anchor retinoschisin to these membranes is not known at the present time. Homology modeling and molecular dynamics simulations of the retinoschisin discoidin domain, however, have led to the suggestion that retinoschisin, like blood coagulation Factors V and VIII, may bind to membrane phospholipids (22). In this study, we have used mass spectrometry based proteomics, biochemical and immunocytological methods to identify components in retinal extracts that interact with retinoschisin. Our studies indicate that retinoschisin does not bind to membrane lipids, but instead forms a complex with Na/K ATPase and SARM1. This interaction implicates retinoschisin in a novel signal pathway important in photoreceptor-bipolar synaptic structure and function and retinal cell organization.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-dioleoyl-sn-glycero-3-phospho-choline (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Purified activated Factor Va and an anti-Factor Va monoclonal antibody were obtained from Hematologic Technologies (Essex Junction, VT). The anti-human SARM1 polyclonal antibody was purchased from Cedarlane Lab (Hornby, ON). Anti-Na/K ATPase \( \alpha 3 \) isoform (MA3–915) monoclonal antibody was obtained from Abcam Inc (Cambridge, MA) and an anti-Na/K ATPase \( \beta 2 \) isoform polyclonal antibody was purchased from Upstate-Millipore (Billerica, MA). The anti-retinoschisin monoclonal antibody R51 3R10 and polyclonal antibody have been described previously (3, 15). Human donor eyes were obtained from the UBC Eye Bank.

**Purification of Retinoschisin**—Wери-retino-blastoma cells (American Type Culture Collection, Manassas, VA) were grown in RPMI media containing 10% fetal calf serum. After 2 weeks, the media containing the secreted retinoschisin was collected, centrifuged at 20,000 \( g \) for 20 min to remove any cell debris, and precipitated with 40% ammonium sulfate at 4 °C. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4, at 1/40 the initial volume of the cell culture media. The solution was dialyzed overnight against 20 mM Tris-HCl, pH 7.4, and centrifuged at 100,000 \( g \) for 10 min to remove residual precipitated protein. For some experiments, retinoschisin was further purified by affinity chromatography on a galactose-agarose column.

The partially purified retinoschisin protein solution obtained after ammonium sulfate precipitation was incubated with 0.2 ml of galactose-agarose at 4 °C. After extensive washing in 20 mM Tris-HCl, pH 7.4, the bound retinoschisin was eluted with 1 M isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) and subsequently dialyzed extensively against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl to remove the IPTG. For some experiments, retinoschisin was extracted and partially purified from bovine retina as previously described (7).

**Preparation of Phospholipid Vesicles**—Lipids were extracted from retinal cell membranes using the method of Bligh and Dyer (25). Briefly, retinal membranes were vortexed in a solution consisting of 1 part chloroform, 2 parts methanol, and 0.8 parts water. Subsequently, a 1:1 mixture of chloroform:water was added and the solution was sonicated in a water bath and centrifuged at 1000 \( g \) for 10 min to induce a phase separation. The lower organic phase containing the lipids was recovered and dried to a homogeneous film under nitrogen.

Large unilamellar phospholipid vesicles were prepared by the method of Hope et al. (26). Synthetic phospholipids (3:1 ratio of DOPC to DOPS, 3:1 ratio of DOPC to DOPE or 1:1:1 ratio of DOPC:DOPS:DOPE) were dissolved in a 1:1 mixture of chloroform:methanol and dried to a homogeneous film under a stream of nitrogen gas. The synthetic phospholipids or retinal lipids were extracted with 20 mM HEPES buffer, pH 7.4, containing 300 mM sucrose and extensively vortexed (27). The resulting multilamellar vesicles were subjected to 5 freeze-thaw cycles in liquid nitrogen to promote lipid hydration and equal transmembrane lipid distribution. The multilamellar vesicles were then extruded 10 times through a 100 nm pore polycarbonate membrane (Whatman, Nucleopore, Middlesex, UK) using a lipid extrusion device (Lipex Biomembranes, Vancouver, BC, Canada) to produce large unilamellar vesicles. Finally, the vesicles were dialyzed against 20 mM HEPES, pH 7.4, 150 mM NaCl, and 2 mM CaCl\(_2\).

**Binding of Factor Va (FVa) and Retinoschisin to Phospholipid Vesicles**—The binding of FVa and retinoschisin to lipid vesicles was carried out in HEPES-buffered saline (20 mM HEPES, pH
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7.4, 150 mM NaCl) containing 2 mM CaCl₂ for 10 min at 25 °C. Vesicles containing a total of 0.6 mg of phospholipids were incubated with 1.2 μg of FVa or retinoschisin in a volume of 50 μl at room temperature for 15 min. The mixture was then centrifuged at 15,000 × g for 15 min in a Beckman TL55 rotor. The supernatant containing unbound protein was retained, and the pellet containing the vesicles was washed by centrifugation with HEPES-buffered saline containing 2 mM CaCl₂ to remove any loosely bound protein. The pellet was resuspended in 50 μl of HEPES-buffered saline, and both the supernatant and pellet fractions were analyzed by SDS gel electrophoresis and Western blotting. Binding studies were reproduced at least three times.

Binding of FVa and Retinoschisin to Immobilized Phospholipids—The binding of FVa or retinoschisin to phospholipids immobilized onto microtiter plates was carried out by a modification of the method of Ortel et al. (28). DOPC or DOPS was dissolved in methanol at a concentration of 0.32 μg/ml and 100 μl was added to each well of a 96-well microtiter plate (Corning). The lipids were allowed to air-dry overnight at room temperature. The wells were then blocked with 200 μl of blocking buffer (5 mg/ml gelatin, 50 mM Tris-HCl, pH 7.2, 150 mM NaCl) for 1 h at 37 °C and subsequently washed three times with wash buffer (10 mM sodium phosphate buffer, pH 7.3, 120 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20). Binding studies were carried out by adding 50 μl of 0.25 μg FVa or retinoschisin in binding buffer (50 mM HEPES, pH 7, 150 mM NaCl, 2.5 mM CaCl₂ and 1% bovine serum albumin) to each well. After 2 h at 37 °C, the wells were washed three times with wash buffer and then incubated with 50 μl of either anti-FVa or anti-retinoschisin RS1 3R10 culture fluid diluted 1:1000 or 1:5, respectively, in blocking buffer for 1 h at 37 °C. The wells were washed with wash buffer and subsequently incubated with 50 μl of horseradish peroxidase conjugated to goat anti-mouse Ig (Sigma) at a dilution 1:200 for 1 h at 37 °C. The wells were washed 3 times and incubated with 100 μl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (0.5 mg/ml) in PBS containing 0.03% hydrogen peroxide. The absorbance at 405 nm was measured in a microtiter plate reader. All samples were run in triplicate.

SDS-PAGE, Western Blot Analysis, and Protein Assays—Samples were denatured in sample mixture containing 10 mM Tris-HCl, pH 6.8, 4% SDS, 20% sucrose, and 4% β-mercaptoethanol and separated on 9% or 10% SDS-polyacrylamide gels. For Western blotting, proteins were transferred onto Immobilon-FL membranes (Millipore, Bedford, MA) using a semi-dry electrophoretic apparatus. The blots were blocked in 1% skim milk in PBS for 30 min and labeled for 1 h with the primary antibody in 0.5% skimmed milk in PBS containing 0.05% Tween 20 (PBS-T) at the recommended dilution. The blots were washed in PBS-T and labeled for 1 h with a secondary anti-mouse or anti-rabbit Ig antibody conjugated to Alexa Fluor 680 (1:40,000) (Molecular Probes, Eugene, OR) or LI-COR IRDye 800 (1:20,000) (Rockland, PA) for analysis on a LI-COR Odyssey imager (Lincoln, NE). Protein concentrations were determined by the BCA method (Pierce).

Preparation of Retinal Membranes—Two bovine or human retinas were incubated in 2.0 ml of TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 1 mM MgCl₂ and Complete protease inhibitor (Roche Applied Sciences, Basel, Switzerland) for 25 min on ice. Retina cells were homogenized using a Dounce homogenizer and subsequently passed through a 22-gauge needle. The homogenate was loaded on top of 50% sucrose in TBS and centrifuged in an Optima TLS55 rotor (Beckman) for 30 min at 26,000 rpm. The retinal membranes, which banded on top of the 50% sucrose, were collected and washed by centrifugation. For the preparation of mouse retinal membranes, 16 retinas from wild-type C57BL/6 or retinoschisin-deficient mice (15) were suspended in 20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, and 1 mM CaCl₂ containing Complete protease inhibitor. The retinas were homogenized by repeated passage through a 22-gauge needle, and the retinal membranes were isolated by centrifugation on a sucrose cushion as described above.

Immunoprecipitation of Retinoschisin from Retinal Membranes—Human and bovine retinal membranes (6 mg) were solubilized in 3.0 ml of 10 mM CHAPS in TBS and mouse retinal membranes (1 mg) were solubilized in 500 μl of 10 mM CHAPS in 20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5% glycerol. The solution was stirred at 4 °C for 20 min and subsequently centrifuged in Optima TLA100.4 rotor (Beckman) for 10 min at 50,000 rpm to remove residual unsolubilized material. Typically, 70% of the total retinoschisin, Na/K ATPase and SARM1 proteins were solubilized from bovine retinal membranes under these conditions. The solubilized extract was applied to 50–100 μl of Sepharose beads conjugated with the RS1 3R10 retinoschisin monoclonal antibody (or an irrelevant monoclonal antibody Rm 3F4 as a control) prepared as previously described (5). The mixture was incubated for 1 h at 4 °C with gentle agitation in an Ultrafilter filter unit (Millipore, Billerica, MA). The beads were washed six times in TBS containing 10 mM CHAPS by low speed centrifugation to remove unbound protein. Bound protein was eluted directly with 2% SDS in TBS or first with 5 mg/ml of the 3R10 competing peptide (LSSTEDEGEDPWYQKA) in CHAPS buffer followed by a second elution with 2% SDS in TBS. The bound protein was collected by low speed centrifugation.

Mass Spectrometry—In-gel digestion of immunoprecipitated proteins was performed by punching out the Coomassie-stained bands from a Nu-Page SDS gradient gel with a Pasteur pipette. The gel pieces were washed with water several times to remove acetic acid and destained in a 1:1 mixture of 100 mM ammonium bicarbonate and 100% acetonitrile several times. After treating with 100% acetonitrile, the gels were dried and subsequently incubated with 10 μl of diithiothreitol followed by 50 μl of iodoacetic acid, washed with ammonium bicarbonate, and dried from 100% acetonitrile. The gel pieces containing the samples were incubated with porcine trypsin (Promega, Madison, WI) at 12 ng/μl in 50 mM ammonium bicarbonate and 5 mM CaCl₂ for 30 min on ice. The protease solution was removed, and the gel pieces were overlaid with 50 mM ammonium bicarbonate and 5 mM CaCl₂. The samples were digested for 18 h at 37 °C. The solution was collected in a separate tube, and the gel pieces were re-extracted with 50 mM ammonium bicarbonate/66% acetonitrile (basic extraction) and with 5% formic acid/66% acetonitrile (acidic extraction). The samples were pooled, dried in a Speedvac, and resuspended in 10 μl of
50 mM ammonium bicarbonate. For MALDI-TOF mass spectrometry, protease-digested samples (2 μl) were applied to H4 chips (Ciphergen, Fremont, CA). The sample was dried and washed with two quick rinses of water before applying 20% α-cyanohydroxycinnamic acid (CHCA) matrix in a solvent with 50% acetonitrile and 0.1% trifluoroacetic acid. Samples were analyzed on a Ciphergen mass spectrometer. Masses obtained were average masses and mined against a mammalian data base using Profound server. A Qstar XL LC/MS/MS (Applied Biosystems, Foster City, CA) was used for MS/MS analysis of peptides. Trypsinized samples obtained as above were lyophilized and reconstituted in formic acid. A PepMap C18 column with a 3-mm particle size and 100-Å pore size column from LC Packings (Amsterdam, Netherlands) was used for peptide separation. Solvents B and A contained 20% acetonitrile in water and 5% acetonitrile in water, respectively. LC conditions started at 2% solvent B, with a gradient to 60% B over 60 min, to 95% B at 93 min, and held for 3 min before returning to 2% B. Masses obtained were monoisotopic masses. Mass spectra were searched against the mammalian data base using Mascot Server v2.1.

**Immunofluorescence Microscopy**—Eyes from 2-month-old C57BL/6 mice were fixed in 4% paraformaldehyde/100 mM phosphate buffer, pH 7.2, containing 5% sucrose for 2 h. The eyes were then washed 3 times with 100 mM phosphate buffer, pH 7.2, (PB) containing 5% sucrose. Eyecups were frozen in Tissue-Tek OCT and 12 μm cryosections were cut. The sections were blocked and permeabilized in 10% goat serum and 0.2% Triton X-100 in PB for 20 min and subsequently labeled overnight at room temperature with the primary polyclonal retinoschisin antibody and monoclonal antibody to Na/K ATPase diluted in PB containing 2.5% goat serum and 0.1% Triton X-100 (1:5000 anti-retinoschisin; 1:200 anti-Na/K ATPase (α3-subunit)). The sections were washed with PB buffer and labeled for 1 h with secondary antibodies conjugated to Alexa-488 or Alexa-594 diluted 1:100 in PB containing 2.5% goat serum and 0.1% Triton X-100. Samples were visualized under a Zeiss Axioplan fluorescence microscope equipped with an Eclipse image analyzer.

**RESULTS**

**Binding of Retinoschisin and FVa to Immobilized Phospholipids**—The C2 discoidin domain of blood coagulation Factor V is known to bind anionic phospholipids and in particular phosphatidylserine (19, 29). This interaction enables the prothrombinase complex to assemble on the surface of platelets as a key reaction in the blood coagulation cascade. Retinoschisin associates with retinal membranes and in particular the extracellular surfaces of photoreceptor and bipolar cells (3, 24). Homology modeling and molecular dynamics simulations of the retinoschisin discoidin domain has led to the suggestion that retinoschisin like Factor V may bind to the phospholipid bilayer through the interaction of conserved hydrophobic residues within the spike region of the discoidin domain (22).

To determine if retinoschisin binds to membrane phospholipids, microtiter plates containing either immobilized DOPS or DOPC were treated with either retinoschisin or FVa (the proteolytically cleaved active form of Factor V) as a positive control, and bound protein was determined by solid-phase ELISA. Fig. 1 shows that FVa binds specifically to DOPS, but not DOPC in agreement with its known phospholipid binding properties (28, 29). In contrast, no significant binding of retinoschisin was observed to either immobilized DOPS or DOPC.

**Binding of Retinoschisin and FVa to Phospholipid Vesicles**—It is possible that retinoschisin only binds to phospholipids organized in a lipid bilayer. This was investigated by measuring the binding of FVa and retinoschisin to phospholipid vesicles. Large unilamellar vesicles consisting of DOPC:DOPE or DOPC:DOPS at a molar ratio of 3:1, and DOPC:DOPE: DOPS at a molar ratio of 1:1:1 were incubated with either retinoschisin or FVa as a control in the presence of calcium. The vesicles were separated from the unbound protein by ultracentrifugation for analysis by Western blotting. Fig. 2A shows that a significant fraction of FVa bound to DOPC:DOPS vesicles, but not DOPC:DOPE vesicles as expected. In contrast retinoschisin did not bind to either DOPC:DOPS or DOPC:DOPE vesicles (Fig. 2B). Likewise, FVa bound to vesicles consisting of an equal mixture DOPC:DOPE:DOPS, whereas retinoschisin did not (data not shown).

As part of this study the interaction of retinoschisin and FVa with unilamellar vesicles composed of retinal membrane lipids was examined. Whereas FVa showed significant binding to retinal lipid vesicles, no binding of retinoschisin was detected (Fig. 2, A and B). Finally, we compared retinoschisin isolated from retina tissue with retinoschisin secreted from Weri-retinoschisin cells for binding to phospholipid vesicles. As shown in Fig. 2C, retinoschisin from either source failed to bind to lipid vesicles.

**Mass Spectrometric Identification of Proteins Co-immunoprecipitating with Retinoschisin**—Because the studies described above strongly indicate that retinoschisin does not bind to membrane lipids, we studied the interaction of retinoschisin with retinal proteins by co-immunoprecipitation. CHAPS-solubilized retinal cell membranes were applied to an anti-retinoschisin immunoaffinity matrix consisting of the RS1–3R10 mono-
clonal antibody from the immunoaffinity matrix upon treatment with SDS. In addition to these proteins, keratin, a common contaminant in proteomic studies, and porcine trypsin used to generate the tryptic peptides were also detected by mass spectrometry. The principal human retinal membrane proteins present in the eluted fraction from the RS1–3R10 immunoaffinity matrix are listed in Table 1.

Western Blots of Retinal Proteins Eluted from the Retinoschisin Immunoaffinity Matrix—Western blotting was used to validate the mass spectrometric identification of proteins that co-elute with retinoschisin on a RS1 3R10 immunoaffinity matrix. Fig. 4A shows Western blots of the retinal membrane extract applied to the immunoaffinity matrix (input lane) and the fraction eluted from the immunoaffinity matrix with SDS (bound lane). The Na/K ATPase α3-subunit, Na/K ATPase β2-subunit, and SARM1 were all detected in the SDS-eluted fraction along with the intensely labeled retinoschisin monomer and a weakly stained dimer.

Additional immunoprecipitation studies were carried out to assess the specificity of the RS1 3R10 immunoaffinity matrix. A retinal extract was added to an immunoaffinity matrix containing the anti-retinoschisin monoclonal antibody (RS1 3R10) or an irrelevant monoclonal antibody (Rim 3F4) as a negative control. After removing unbound protein, the bound proteins were eluted first with the 3R10 competing peptide under non-denaturing conditions and subsequently with SDS under denaturing conditions. Approximately, a third of the retinoschisin and NaATPase α3- and β2-subunits that bound to the RS1 3R10 matrix were eluted with 3R10 peptide. The remaining bound protein was eluted with SDS. In contrast, retinoschisin and Na/K ATPase β2 were not detected in the eluted fractions from the control Rim 3F4 immunoaffinity matrix and only faint labeling of the Na/K ATPase α3-subunit was observed. Likewise, SARM1 specifically bound to the RS1 3R10 matrix and was not detected in the eluted fraction from the control Rim 3F4 column (data not shown).

Retinoschisin Is Required for the Immunoprecipitation of Na/K ATPase on a RS1 3R10 Immunoaffinity Matrix—Na/K ATPase is a highly abundant membrane protein in the retina and in particular photoreceptor cells (30). Therefore, it is possible that co-elution of Na/K ATPase with retinoschisin could be due to residual interaction with the RS1 3R10 immunoaffin-
Co-localization of Na/K ATPase with Retinoschisin and SARM1 in Retina Tissue—Immunofluorescence microscopy was used to visualize the distribution of retinoschisin, Na/K ATPase (α3) and SARM1 in retinal cryosections of adult mice. Intense immunolabeling of retinoschisin and Na/K ATPase was observed in photoreceptor inner segments, outer plexiform layer and inner nuclear layer of the retina (Fig. 6, A and B). Less intense staining was seen within the outer nuclear and inner plexiform layers. Merged images confirmed a high degree of colocalization of the Na/K ATPase and retinoschisin in the various retinal layers (Fig. 6C). Retina tissue was also labeled with a commercial antibody to SARM1. The labeling pattern was similar to that of Na/K ATPase and retinoschisin with labeling in the inner segment, outer nuclear and plexiform layers of the outer retina and inner nuclear and inner plexiform layers of the inner retina (Fig. 6E). Double labeling studies indicated that SARM1 co-localized with Na/K ATPase in these layers (Fig. 6, D–F). In addition the SARM1 antibody labeled the retinal pigment epithelium and ganglion cells consistent with the general finding that SARM1 is widely distributed in most cells. In control experiments, no labeling was observed when the retinoschisin, Na/K ATPase, or SARM1 primary antibodies were omitted in the labeling procedure (data not shown). As a cautionary note, the only commercial SARM1 antibody available for this study labeled a number of protein bands in Western blots of retinal extracts (Fig. 4A). Accordingly, it is possible that some of the immunofluorescence labeling observed with the SARM1 antibody is nonspecific in nature. We are in the process of developing additional SARM1 antibodies to further evaluate the localization of SARM1 in retina tissue.

DISCUSSION

To gain insight into the molecular and cellular mechanisms underlying the role of retinoschisin in the normal retina structure and function and the pathogenesis of XLRS, we examined the interaction of retinoschisin with retinal membrane lipids and proteins. Our studies provide strong evidence that retinoschisin is not bound to photoreceptor and bipolar surface membranes through lipids, but instead is anchored to these cells through its interaction with a protein complex consisting of the Na/K ATPase and SARM1.

Initially, we examined the possibility that retinoschisin may interact with membrane lipids through its discoidin domain analogous to the binding of blood coagulation proteins Factor V and VIII to phosphatidylserine rich regions on blood platelets. Our results indicate that retinoschisin does not bind to either synthetic phospholipid mixtures or retinal lipids immobilized on microtiter plates or organized in lipid vesicles, whereas FVa specifically interacts with phosphatidylserine in these systems as previously reported (28, 29). Interestingly, during our studies, Vijayasrathy et al. (31) published a report suggesting that the discoidin domain of retinoschisin has a strong affinity for anionic phospholipids including phosphatidylserine. Evidence for this protein-lipid interaction was based on the observation that GST fusion proteins containing wild-type and mutant retinoschisin discoidin domains bound to commercial nitrocellulose strips containing anionic lysophospholipids. A number of aspects of this study, however, raise concerns on the validity of this investigation. First, the nitrocellulose strips used in these lipid-protein overlay experiments are prone to nonspecific binding artifacts as stated by the manufacturer of these strips. Accordingly, the manufacturer recommends that other techniques should be used to validate the interactions observed with these strips. This was not done in their study. Second, bacterial-expressed GST fusion proteins containing only the retinoschisin discoidin domain were used in these lipid-protein overlays instead of the native retinoschisin homo-octameric complex as used in the current study. It is unlikely that the discoidin
domain of retinoschisin folds properly in the reducing environment of *Escherichia coli* and forms multiple intramolecular disulfide bonds needed to stabilize its native structure. Indeed, the biochemical properties of the GST fusion proteins were not examined. This is a particular concern since several of the disease-associated discoidin domain mutants used in this study were reported to bind the same anionic phospholipids as the wild-type discoidin domain (31), despite the fact that these mutant proteins have been reported to be highly misfolded when expressed in eukaryotic cells (13). Thus, it is conceivable that the bacterial-expressed GST fused discoidin domains are not properly folded and therefore cannot be used as model systems to investigate the binding properties of native and mutant retinoschisin. Our data showing that native octameric retinoschisin does not bind to either immobilized lipids or lipids organized in a bilayer together with the potential limitations in using GST-discoidin domain fusion proteins and lysophospholipid strips argue against retinoschisin being anchored to cell surfaces through its interaction with membrane lipids.

On the other hand, our studies provide compelling evidence for the anchoring of retinoschisin to photoreceptor and bipolar cell surfaces through protein-protein interactions. When a retinal membrane extract solubilized in CHAPS detergent was applied to an anti-retinoschisin immunoaffinity matrix, two prominent proteins, the Na/K ATPase and SARM1, co-eluted with retinoschisin as determined by mass spectrometry-based proteomic analysis and confirmed by Western blotting. Control experiments support the specificity of interaction between these proteins and retinoschisin. First, neither Na/K ATPase nor SARM1, was detected in the SDS-eluted fraction from an immunoaffinity matrix in which the anti-retinoschisin RS1 3R10 monoclonal antibody was replaced with an irrelevant monoclonal antibody. Second, when retinal membrane extracts from retinoschisin-deficient mice were applied to the RS1 3R10 immunoaffinity matrix, neither Na/K ATPase nor SARM1 were detected in the SDS-eluted fraction, indicating that retinoschisin is required for co-precipitation of these proteins. Finally, a significant fraction of Na/K ATPase co-eluted with retinoschisin when the anti-retinoschisin RS1 3R10 immunoaffinity column was treated with the competing 3R10 peptide under mild, nondenaturing conditions.

Na/K ATPase is the prime candidate for directly anchoring retinoschisin to the surface of photoreceptor and bipolar cells. Na/K ATPase, an integral membrane protein found on the plasma membrane of essentially all eukaryotic cells, is composed of two principal subunits (α and β), organized as a heterodimer (32). A third small γ-subunit is found in many tissues.
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The α-subunit containing multiple transmembrane segments and large cytoplasmic regions is the catalytic subunit responsible for the ATP-dependent transport of Na and K across membranes. The β-subunit with a single transmembrane segment plays a role in the assembly of the complex, targeting to specific cell membranes and modulation of the functional properties of the catalytic subunit. Both subunits have multiple variants with four well-characterized α-subunit isoforms (α1, α2, α3, α4) and three β-subunit isoforms (β1, β2, β3). In the present study, the α3 and β2 isoforms were detected in the eluted fraction from a retinoschisin immunoaffinity matrix as determined by mass spectrometry and confirmed by Western blotting. These isoforms have been previously shown to be expressed in photoreceptor and bipolar cells and distributed in the inner segment, outer nuclear and outer plexiform layers of the outer retina and the inner nuclear layer containing the bipolar cells of the inner retina in adult mouse and rat (30). In the present study, we have shown that retinoschisin and Na/K ATPase exhibit a remarkably similar pattern of labeling with intense staining in the inner segment, outer plexiform and inner nuclear layers and more moderate staining in the outer nuclear and inner plexiform layers. Merged images of Na/K ATPase α3 and retinoschisin immunolabeling further confirmed the co-localization of these proteins in the retina. Likewise, SARM1 co-localizes with Na/K ATPase α3 over much of the retina, consistent with its participation in this complex.

Retinoschisin most likely binds to the β2-subunit of Na/K ATPase. This subunit contains a large extracellular domain with up to nine glycosylation sites and as a result migrates on SDS gels abnormally with an apparent mass of ~50 kDa instead of the predicted protein molecular mass of 33 kDa (33). Retinoschisin, like other discoidin domain containing proteins, has an affinity for galactose (34). Therefore, it is possible that the oligosaccharide chains of the β2-subunit may contribute to retinoschisin binding, although this remains to be determined. The α3-subunit of Na/K ATPase contains only small extracellular loops joining the transmembrane segments and therefore is less likely to contain a binding site for retinoschisin. Interestingly, the β2-subunit, also known as AMOG (Adhesion Molecule on Glia), has been implicated in neural cell adhesion (33, 35).

In addition to Na/K ATPase, SARM1, also known as sterile alpha

4 R. S. Molday, unpublished data.
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and TIR motif-containing protein 1, was present in the fraction that co-eluted with retinoschisin from the anti-retinoschisin immunoaffinity matrix. SARM1 is a 690-amino acid protein containing two sterile alpha motif (SAM) domains flanked by short HEAT/armadillo repeat sequences and a Toll/interleukin-1 receptor (TIR) domain (36). These domains have been found in a number of membrane and cytoplasmic proteins and have been implicated in protein-protein interactions. SARM1 lacks membrane-spanning segments as analyzed by membrane topology prediction programs including TMHMM or SOSUI, and therefore, is unlikely to directly interact with retinoschisin.

The function of SARM1 is not well understood, but a recent report suggests that it interacts with and negatively regulates TRIF, an Toll/interleukin-1 receptor (TIR)-domain-containing adaptor protein that controls interferon-β production as part of the Toll-like receptor (TLR) signaling pathway associated with innate immunity (37, 38). The interaction of SARM1 with TRIF occurs through the SAM and TIR domains of SARM1. Our studies suggest that SARM1 may function as a cytoplasmic adaptor protein in a novel retinal cell signaling pathway. SARM1 most likely binds to a site within the cytoplasmic domains of the α3- and/or β2-subunits of Na/K ATPase, possibly through its SAM and TIR domains. Alternatively, it could bind to another “adaptor” protein that interacts with Na/K ATPase, although such a candidate protein has not been detected in our proteomic studies.

Retinoschisin is a homo-octameric complex in which proximal subunits are linked together through intermolecular disulfide bonds between cysteine residues Cys99 and Cys223 (7). Replacement of either of these cysteine residues with serine or other amino acids results in essentially normal retinoschisin expression and secretion, but defective octamerization (5, 7). The functional importance of octamer formation is highlighted by the finding that C59S and C223R mutations in retinoschisin known to prevent octamer formation (5) are associated with XLRS (11, 39, 40). What role does the octameric structure of retinoschisin play? Based on our current studies, we propose a model in which retinoschisin binds to an extracellular site on the Na/K ATPase β2-subunit, possibly involving oligosaccharide chains (Fig. 7). Through its multi-valency, retinoschisin promotes the oligomerization of the Na/K ATPase in the plasma membrane. Oligomerization could induce a protein conformational change allowing SARM1 to bind Na/K ATPase on the cytoplasmic side of the plasma membrane. SARM1 may interact with other proteins including cytoskeletal proteins as part of a cell signaling process that is crucial for maintaining normal photoreceptor-bipolar synaptic structure and function and stabilizing interactions between the extracellular matrix and photoreceptor and bipolar cell surfaces. The essence of this model is that retinoschisin does not directly interact with components of the extracellular matrix, but instead affects photoreceptor and bipolar cell structure, function, and organization indirectly via intracellular signaling events important in cell structure.

In another possible model, extracellular proteins could directly interact with the retinoschisin-Na/K ATPase-SARM1 oligomeric complex thereby anchoring the extracellular matrix to photoreceptors and bipolar cells and stabilizing the cellular and synaptic organization of the retina. In a preliminary study Steiner-Champliaud et al. (41) reported that β2 laminin co-precipitated with retinoschisin on an immunoaffinity column. However, β2 laminin showed only limited overlapping distribution with retinoschisin in retina tissues by immunofluorescence microscopy. Furthermore, we have not observed β2 laminin in our co-immunoprecipitation studies using highly sensitive and specific proteomic methods as reported here. Accordingly, further studies are needed to determine if β2 laminin or other extracellular proteins directly interacts with retinoschisin-Na/K ATPase complex.

In summary our studies indicate that retinoschisin is anchored to the surface of photoreceptor and bipolar cells through its interaction with a multi-protein complex containing the Na/K ATPase (α3-, β2-subunits) and SARM1. This complex may be part of a novel signaling pathway important for the structure and function of the photoreceptor-bipolar synapse and normal interaction of these cells with the extracellular matrix. Disease-causing mutations in retinoschisin resulting in a nonfunctional protein would disrupt this signaling pathway leading to the destabilization of the retinal cell organization and loss in photoreceptor-bipolar synaptic structure and signal transmission as is evident in patients with XLRS and mice deficient in retinoschisin. Studies are now in progress to further define the structural basis for the retinoschisin-Na/K ATPase interactions and components of the signal transduction pathway linked to SARM1.

Acknowledgments—We thank Dr. Leonard Foster for help with MS/MS data analysis, Dr. Kim Wong for technical help with the preparation of phospholipid vesicles, Dr Bernhard Weber for the retinoschisin-deficient mice, and Dr. Frank Dyka for helpful discussions.

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