

# Retinoschisin Is a Peripheral Membrane Protein with Affinity for Anionic Phospholipids and Affected by Divalent Cations

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**PURPOSE.** Retinoschisin (RS) is a retina-specific, secreted protein implicated in X-linked juvenile retinoschisis and essential for the structural and functional integrity of the retina. This biochemical characterization and ultrastructural localization of RS in intact murine retina was performed to further understanding of the molecular basis of its function.

**METHODS.** Subcellular fractions and fractions enriched in photoreceptor inner and outer segments were prepared from mouse retina by differential or density gradient ultracentrifugation. Immunoblot analysis was used to assess the expression of RS in various subcellular compartments and its fractionation into soluble phase on treatment of retinal cell membranes with several solubilizing reagents. RS-lipid interactions were evaluated by a protein-lipid overlay assay that used wild-type and mutant forms of RS discoidin domain glutathione S-transferase (GST) fusion proteins. The subcellular localization of RS in mouse retina was visualized by pre-embedding immunogold electron microscopy. Ultrastructure was evaluated by transmission electron microscopy.

**RESULTS.** RS was intimately associated with cell membranes of the retina. It was found to cluster on the outer leaflet of the plasma membrane of the photoreceptor inner segments, which synthesize and secrete it. It was released from the membrane at high pH, which is characteristic of a peripheral membrane protein. It was extracted from the membrane by the nonionic detergent NP-40, together with glycerophospholipids. Protein-lipid overlay assays indicated a preferential interaction between RS and anionic phospholipids. Extraction of RS from the membrane was inhibited by divalent cations. Photoreceptor inner segment morphology was markedly affected in *RS*<sup>-/-</sup> mice, which failed to express RS protein.

**CONCLUSIONS.** RS in intact retina is a peripheral membrane protein. Although distributed over the two membrane faces, RS is associated primarily with the outer leaflet of the inner segment plasma membrane through anionic phospholipids and divalent cations. RS's localization in photoreceptors and its biochemical properties suggest a functional role locally, at the

site of secretion and membrane adhesion, in maintaining the photoreceptor inner segment stability and architecture. (*Invest Ophthalmol Vis Sci.* 2007;48:991-1000) DOI:10.1167/iovs.06-0915

Retinoschisin (RS) is a 24-kDa secreted protein that is expressed exclusively in the retina<sup>1</sup> and pineal gland.<sup>2</sup> It is believed to function as an adhesive protein in preserving the structural and functional integrity of the retina.<sup>3</sup> The mutations in the *RS1* gene that encodes this protein cause X-linked retinoschisis (XLRs).<sup>1</sup> XLRs is a form of juvenile macular degeneration that affects males and causes schisis or splitting within the retinal layers that leads to early and progressive loss of vision.<sup>4,5</sup> Currently there is no treatment for XLRs. The feasibility of gene therapy as an approach to treating retinoschisis was indicated by AAV-mediated delivery of the *RS1* gene into *RS1*<sup>-/-</sup> mice, which restored the electrophysiological waveform properties to that of the normal retina.<sup>6</sup>

RS is synthesized by retinal photoreceptors and other retinal neurons, and after the cleavage of the N-terminal signal sequence (amino acids 1-23), it is secreted into the extracellular space.<sup>7-9</sup> A recent study identified two mature forms of RS in murine retina that differed by two amino acids at the N terminus.<sup>10</sup> Processing of RS signal sequence at two cleavage sites (between amino acids 21-22 and 23-24) by signal peptidase was suggested as the basic mechanism underlying their occurrence in vivo. RS exists as a disulfide-linked homo-octamer.<sup>11</sup> Because it is distributed throughout the retina, it has been proposed that RS is secreted from photoreceptor inner segments and is transported by Müller cells into the inner retina.<sup>9,12</sup> However, our previous studies demonstrated that all major classes of adult retinal neurons have RS message and express the protein, suggesting that RS is synthesized locally by neurons, even in the inner retina.<sup>13</sup>

A conserved discoidin domain (DD) sequence of 155 to 160 amino acids comprises most of the RS molecule.<sup>1</sup> Members of the DD family of proteins are involved in cell adhesion and cell-cell interactions.<sup>14</sup> Although previous studies have shown that RS is associated with the membrane fraction of retinal homogenates,<sup>7</sup> neither the biochemical nature of RS association with the membrane nor its organization in the retina at the ultrastructural level has been reported. In this study, we used biochemical and molecular biology methods to describe the nature of RS-membrane associations and to understand the molecular mechanism by which RS maintains the structural and functional integrity of the retina. RS was localized on photoreceptors by immunoelectron microscopy, and the ultrastructural features of photoreceptors in *Rs1* knockout (*RS1*<sup>-/-</sup>) mice were visualized with electron microscopy. Results demonstrate that RS is a peripheral membrane protein that is bound to anionic phospholipids on the outer leaflet of the plasma membrane. Divalent cations influence RS association with the membrane. The biochemical properties of RS and its localization on the inner segment plasma membrane suggest that it has a functional role locally at the site of secretion and that asso-

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ciation with the membrane is necessary for maintaining the stability and architecture of the photoreceptor inner segment.

## MATERIALS AND METHODS

### Animals

C57BL/6J (wild-type) mice and *RS1*<sup>-/-</sup> mice<sup>6</sup> were used in this study. All animal experimental procedures were conducted in accordance with the National Institute of Health (NIH) Animal Care and Use Committee protocols and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Antibodies and Reagents

The rabbit polyclonal RS antibody was raised against a synthetic peptide corresponding to the amino acid residues 24-37 of RS.<sup>13</sup> The following antibodies were also used: human factor VIII; lactase dehydrogenase (LDH); the  $\alpha$  subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase; rhodopsin; collagen types I, IV and XVII (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-collagen type II and III (Sigma-Aldrich, St. Louis, MO); anti-Cyt Oxi IV (MitoSciences, Eugene, OR); non-NMDA receptor subunit I (gift from Robert Wenthold, NIDCD/NIH); flotillin 1 (BD Biosciences, Franklin Lakes, NJ), and anti-glutathione S-transferase (GST) (GE Healthcare/Life Sciences, Piscataway, NJ). Reagents were collagens, human lung type I, bovine type II and III (Southern Biotech, Birmingham, AL); mouse collagen IV (Cultrex; Trevigen, Gaithersburg, MD); Igepal CA630/NP40, glutathione, isopropyl- $\beta$ -D-thiogalactoside, imidazole, phospholipase C from *Bacillus cereus* (Sigma-Aldrich); PIP Strips (Echelon Biosciences, Salt Lake City, UT); glutathione-Sepharose, chemiluminescent Western blot detection reagent, and secondary horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (GE Healthcare/Life Sciences); phospholipids (Avanti Polar Lipids Inc., Alabaster, AL); Factor VIII (Bayer Health Care, Leverkusen, Germany); and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). Analytical grade reagents were obtained from Sigma-Aldrich, Bio-Rad Laboratories (Hercules, CA), or Electron Microscopy Sciences (Hatfield, PA).

### Subcellular Fractionation

Protease inhibitor cocktail was included in all the isolation and incubation media. Retinas from 8- to 10-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were homogenized in three volumes of ice-cold HME buffer (20 mM HEPES [pH 7.4], 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and protease inhibitor cocktail). The homogenates were differentially centrifuged to isolate membrane and cytosolic fractions. The photoreceptor rod outer segments and a fraction enriched in rod inner segments were isolated after a discontinuous sucrose density gradient centrifugation.<sup>15</sup> The rod outer segment (32%-37% sucrose interphase) and inner segment (37%-42% sucrose interphase) enriched fractions were collected and resuspended in 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and protease inhibitor cocktail. The purity of isolated fractions was assessed by immunoblot analysis of the following marker proteins: rhodopsin (rod outer segments), cytochrome *c* oxidase subunit IV (Cyt Oxi IV-mitochondria/photoreceptor inner segment), LDH (cytosol), and the  $\alpha$  subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase (plasma membrane). Protein concentrations of samples were determined by a colorimetric method with the bicinchoninic acid (BCA) kit (Pierce Biotechnology, Inc., Rockford, IL).

### Membrane Extraction

Alkaline and high salt extraction of membrane fractions was performed essentially as described earlier.<sup>16</sup> The membrane fractions (100  $\mu$ g protein) were adjusted to 50 mM Na<sub>2</sub>CO<sub>3</sub>, 1 M KI, 0.3 M KCl, and 1 M KCl by the addition of respective stock solutions of 1 M Na<sub>2</sub>CO<sub>3</sub>, 2 M KI (freshly made in 50 mM Tris-HCl [pH 7.5]), and 3 M KCl and incubated on ice for 15 minutes with occasional vortexing. To pellet the insoluble fraction, the mixture was layered over 0.6 M sucrose and

centrifuged at 100,000g for 30 minutes (TLA 100.2; Beckman, Fullerton, CA). The pellets were solubilized in SDS sample buffer, and the supernatants were precipitated in trichloroacetic acid and solubilized in SDS sample buffer. The pellets and the supernatant fractions were adjusted to equal volumes and analyzed for RS by SDS-PAGE followed by immunoblot analysis.

### Protease Protection Assays

For protease protection assays, freshly prepared microsomal membrane fractions (80-100  $\mu$ g protein in 320 mM sucrose and 10 mM HEPES [pH 7.4]) were adjusted to 10 mM CaCl<sub>2</sub> and incubated at 0°C for 40 minutes with various amounts of trypsin and chymotrypsin (1, 3, 5, and 10  $\mu$ g each), with or without 1% Triton X-100 present.<sup>17</sup> After 30 minutes, 40  $\mu$ g of aprotinin was added to each sample, and incubation was continued for 5 minutes on ice. After ultracentrifugation (100,000g for 30 minutes) the protease-resistant membrane fractions recovered in pellets were analyzed for RS by SDS-PAGE and immunoblot analysis. The integrity of the membranes and the validity of the assay was confirmed by reprobing the blots with the following antibodies directed against the membrane-oriented protein epitopes: H-300, a polyclonal antibody raised against amino acids 551-850 mapping within the transmembrane and cytosolic region of Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$  (Santa Cruz Biotechnology, Inc.), and APC-035, a polyclonal antibody raised against the C terminus amino acids 356-375 of Kir4.1 (Alomone Laboratories, Ltd., Jerusalem, Israel).

### Extraction of Membranes with NP-40 and Triton X-114 Phase Separation

Aliquots (100- $\mu$ g) of the membrane proteins in 200  $\mu$ L buffer (320 mM sucrose, 10 mM HEPES [pH 7.4] plus protease inhibitor cocktail) containing different concentrations of NP-40 were incubated for 10 minutes at 25°C.<sup>18</sup> After high-speed centrifugation at 30,000g for 20 minutes, the detergent-insoluble and soluble phases were separated and adjusted to equal volumes, and the amount of RS in each fraction was determined by immunoblot analysis. The Triton X-114 phase separation was performed essentially as described by Bordier.<sup>19</sup> The membrane pellets prepared from the sucrose density gradient sedimentation were diluted to 2 mg protein/mL in 10 mM-HEPES-NaOH (pH 7.4) and precondensed Triton X-114 was added to a final concentration of 2% in a total volume of 0.2 mL. Detergent-insoluble material was removed by centrifugation at 16,000g for 15 minutes at 4°C, and soluble fractions were subjected to aqueous and detergent phase separation at cloudy point temperature above 20°C. Integral proteins partition into the detergent phase, whereas most peripheral proteins go into the aqueous phase. The two phases were subjected to one round of washing, and the volumes were adjusted to the original volume of the extract. Equal volumes were used for SDS/PAGE and immunoblot analysis.

The effects of divalent cations on RS extraction were probed by using freshly isolated retinal cell membranes (100  $\mu$ g protein in 160 mM Tris-HCl [pH 7.4] or PBS [pH 7.4]) incubated for 60 minutes at 37°C in the absence or presence of divalent cations or chelating agents. After centrifugation at 100,000g for 15 minutes, the soluble fractions were analyzed for RS by SDS-PAGE and immunoblot analysis. To assess the effect of divalent cations on NP-40 extraction of RS, the membranes were first preincubated for 10 minutes on ice in various buffers in the presence or absence of Ca<sup>2+</sup> or Mg<sup>2+</sup>. After the addition of NP-40 (final concentration 0.2%), the samples were further incubated for 10 minutes at 25°C. The incubation medium was centrifuged, and the RS released into the supernatant was analyzed by immunoblot analysis. The experimental conditions for PLC treatment and divalent cation effects are described in the corresponding figure legends.

### Thin Layer Chromatography

The pellet and supernatant fractions obtained after extraction of the retinal membranes with 0.1% NP-40 were extracted in a 1:1 mixture of chloroform and methanol for 10 minutes on ice. After the addition of

0.25 mL of water, the emulsion was centrifuged for 5 minutes. The lower organic phase was recovered and dried under nitrogen. The samples were dissolved in chloroform, and equal volumes were subjected to lipid analysis by thin-layer chromatography (TLC) on pre-coated silica gel H plates (Analtech Inc., Newark, DE) using the chloroform-methanol-acetic acid-water solvent system (volumes, 50:37.5:3.5:2).<sup>20</sup> Lipids were visualized by exposing the plate in an iodine chamber. Standard lipids for each class were spotted on the same plate for comparison and identification of lipids in the samples.

### Immunoprecipitation, SDS-PAGE, and Western Blot

Whole-cell lysates were prepared from mouse retina by the freeze-thaw method in a lysis buffer (10 mM Tris-HCl [pH 7.4] 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 0.5% Igepal CA 630, plus protease inhibitor cocktail). Whole-cell extracts or membrane fractions representing an equal amount of protein were resolved by 10% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories). Blots were incubated with appropriate primary and secondary antibodies followed by chemiluminescent detection (Super Signal West Dura; Pierce Biotechnology). The blots were scanned and signals quantified (Image Station 2000R; Eastman Kodak, Rochester, NY) For reprobing the blots were stripped in Western blot stripping buffer (Restore; Pierce Biotechnology). Immunoprecipitation was performed overnight in 0.5 mL lysis buffer containing 500  $\mu$ g total cell extract and 2 to 4  $\mu$ g of the respective antibodies. The immunocomplexes were captured by protein A agarose beads, and profiles of the proteins pulled down by the antibodies were analyzed by Western blot analysis.

### GST-RS Discoidin Domain Fusion Constructs, Site-Directed Mutagenesis, and GST Fusion Proteins

The primer pair with the *EcoRI* or *XbaI* restriction sites 5'-ggaattc TGCCCATATCACAAAGCCCCTG and 5'-gctcgag TCAACACTCAAG-CAGCTCCATCCG was used to amplify the discoidin domain region (amino acids 63-219) of mouse RS. The amplified fragment was cloned into the *EcoRI-XbaI* sites of a cloning vector (pCR-Blunt II-TOPO; Invitrogen, Carlsbad, CA). For production of recombinant mouse RS discoidin domain fused to GST, the discoidin domain insert from TOPO vector was subcloned into *EcoRI-XbaI* sites of vector (pGEX 6P1; GE Healthcare/Life Sciences). Correct fusion of this construct and the mutant constructs were confirmed by automated DNA sequencing. Wild-type mouse RS discoidin domain (RS-WT) was expressed in *Escherichia coli* strain BL21 after transformation of the expression plasmid, using standard methods recommended by the manufacturer (GE Healthcare/Life Sciences). Replacement of Tyr-89 (Y-89), Trp-92 (W92), and Phe-108 (F108) residues with Cys (C) was performed by using a site-directed mutagenesis kit (QuikChange; Stratagene, La Jolla, CA) and appropriate primers. The pGEX-6P constructs encoding the WT and mutant versions of the RS discoidin domain were transformed into BL21 *E. coli* cells (Invitrogen). The growth of *E. coli* cells, induction by isopropyl- $\beta$ -D-thiogalactoside, and harvesting and purification of GST recombinant proteins on glutathione-Sepharose columns were performed according to the manufacturer's protocol (GE Healthcare/Life Sciences).

### Lipid-Protein Overlay Assay

To assess the lipid-binding properties of RS, we performed a protein-lipid overlay assay with recombinant GST fusion proteins encoding wild-type RS discoidin domain sequence or its mutant versions (RS-WT). A nitrocellulose membrane with immobilized phospholipids (PIP Strips; Echelon Biosciences) was blocked for 1 hour in 3% (wt/vol) fatty acid-free BSA in TBST (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl, and 0.1% [vol/vol] Tween 20). It was then incubated overnight at 4°C with gentle stirring in the same solution containing either 200 ng/mL of purified wild-type RS or mutant versions of RS discoidin domain encoding GST fusion proteins. The membranes were later incubated

for 1 hour with a 1:2000 dilution of anti-GST antibody followed by a 1-hour incubation with 1:5000 dilution of anti-rabbit-HRP conjugate. The washing steps were repeated between all incubation steps and before detection by chemiluminescence. The binding of positive controls (GRIP and factor VIII) to lipids was detected by using anti-GST or anti-factor VIII monoclonal antibodies followed by a 1-hour incubation with 1:5000 dilution of anti-mouse-HRP conjugate.

### Fractionation of Lipid-Rich and Other Plasma Membrane Domains

Retinas were homogenized in ice-cold isolation medium (150 mM NaCl, 1 mM DTT, and 25 mM Tris-HCl [pH 7.4]) in the presence of either 5 mM EDTA (minus  $\text{Ca}^{2+}$ ) or 5 mM  $\text{Ca}^{2+}$  and centrifuged at 1000g for 10 minutes. The supernatant was adjusted to 1% Triton X-100 and left on ice for 30 minutes. The lysate was later adjusted to 40% iodixanol (OptiPrep; Axis-Shield, Norton, MA) and 0.8 mL of it was placed at the bottom of the 4-mL tubes of a swinging-bucket rotor (SW 60 Ti; Beckman Coulter) and overlaid with 0.8 mL of 35%, 30%, 25%, and 20% iodixanol in the isolation medium. The tubes were spun at 160,000g for 5 hours at 4°C. Sixteen gradient fractions of 250  $\mu$ L each were collected from the top to the bottom of the tube and analyzed for RS and flotillin by Western blot.

### Electron Microscopy

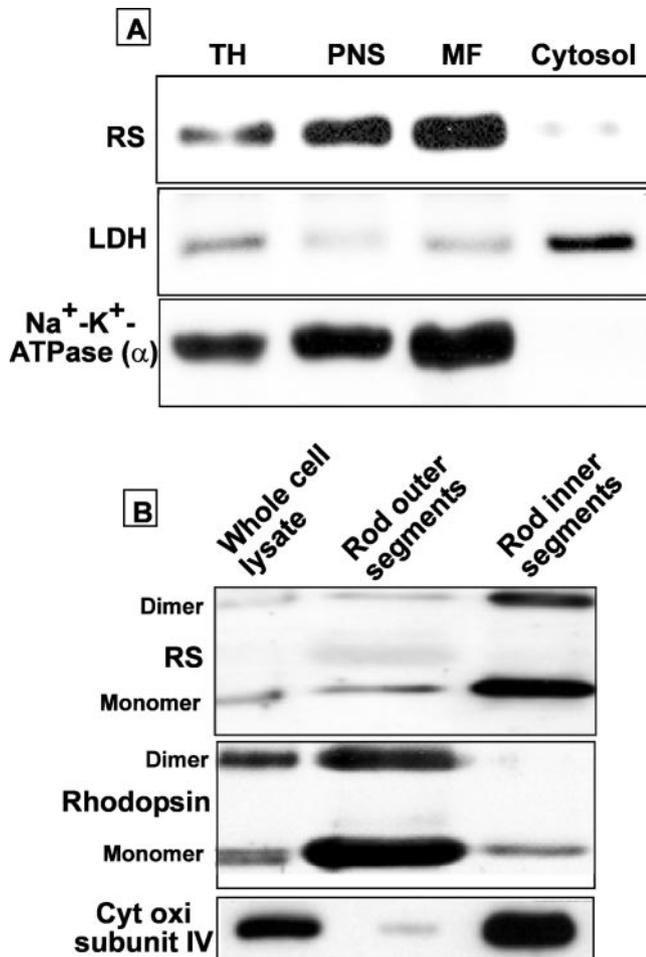
Mice were deeply anesthetized with ketamine (50 mg/kg) and xylazine (5mg/kg) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Eyes were enucleated and hemisectioned, and the posterior eye cup segments were immersed in fixative for 2 hour and rinsed in phosphate-buffered saline (PBS) at 4°C overnight. For transmission electron microscopy, the fixed eye cups were dehydrated in ethanol in series (30%, 50%, 70%, and 96%), block-stained in 1% uranyl acetate in absolute ethanol for 1 hour, rinsed in 2 $\times$  absolute ethanol and embedded via propylene oxide in epoxy resin (Embed 812; Electron Microscopy Science). Ultrathin sections were cut and poststained in uranyl acetate and lead citrate. For immunoelectron microscopy, wild-type eye cups were embedded in 5% agarose/PBS and sectioned at 50- $\mu$ m thickness on a microtome (Vibratome, St. Louis, MO) and then processed for pre-embedding immunoelectron microscopy. Microtome-cut sections were preincubated in 10% normal goat serum (NGS) diluted in PBS for 2 hours and then incubated for 48 hours at 4°C in an anti-RS antibody diluted in PBS-1% NGS. After this the sections were incubated in a mixture of goat anti-rabbit affinity-purified Fab fragment coupled to 1.4-nm gold (1:100; Nanoprobes Inc., Stony Brook, NY). Gold particles were enhanced by silver amplification for 8 to 12 minutes (HQ Silver ki; Nanoprobes). Sections were treated with 1%  $\text{OsO}_4$  and contrasted in 1% uranyl acetate before embedding (Embed 812; Electron Microscopy Science). Serial electron microscopic sections were cut and collected on polyvinyl formal-coated copper slot grids and observed by electron microscope (JEOL, Tokyo, Japan).

All experiments were performed in triplicate, and representative results are presented.

## RESULTS

### RS in Retinal Photoreceptor Inner Segments and Membrane Fractions

The Western blot analysis of subcellular fractions isolated from retina using the RS antibody showed that the membrane fractions abundant in plasma membrane marker protein  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase are highly enriched for RS protein (Fig. 1A). Although RS is known to contain a secretory motif, it was absent in the LDH-enriched cytosolic fraction. These results indicate that mature RS is a plasma membrane-associated protein and not a soluble protein. Photoreceptor inner segments are abundant in mitochondria and were labeled strongly with anti-cytochrome



**FIGURE 1.** RS is mainly membrane associated. Subcellular fractions (A) and fractions enriched in photoreceptor outer and inner segments (B) were isolated from mouse retina and subjected to SDS-PAGE on 10% gels followed by immunoblot analysis using anti-RS antibody. The blots were stripped and reprobbed with antibodies to organelle-specific proteins. The apparent molecular weight of the proteins was: LDH, 36 kDa;  $\alpha$  subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase, 113 kDa; cytochrome oxidase subunit IV (Cyt oxidase subunit IV), 16 kDa; rhodopsin monomer 30 kDa and dimer, 60 kDa; and RS monomer 26 kDa and dimer 55 kDa. TH, total homogenate; PNS, postnuclear supernatant; MF, membrane fraction.

c oxidase subunit IV. Anti-RS antibody strongly labeled this fraction as well (Fig. 1B). Rod outer segments, which are enriched in rhodopsin, showed negligible level of RS expression, confirming the absence of RS immunolabeling of outer segments reported previously.<sup>13</sup> The traces of RS in outer segments and rhodopsin in inner segment fractions reflect slight cross-contamination of the preparations during the isolation procedure.

### RS in the Peripheral Membrane

Proteins were extracted with various solubilizing reagents under conditions in which the membranes remained intact, and the release of RS from the membranes was followed by immunoblot analysis with the anti-RS antibody. RS protein was detected in the soluble fraction after the membrane fractions were treated with 50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 11) and subjected to high-speed centrifugation at 100,000g (Fig. 2A). As is characteristic of many peripheral proteins, most of the RS was released at high pH. Only 50 mM Na<sub>2</sub>CO<sub>3</sub> was effective in dissociating the RS protein from the membrane. On treatment

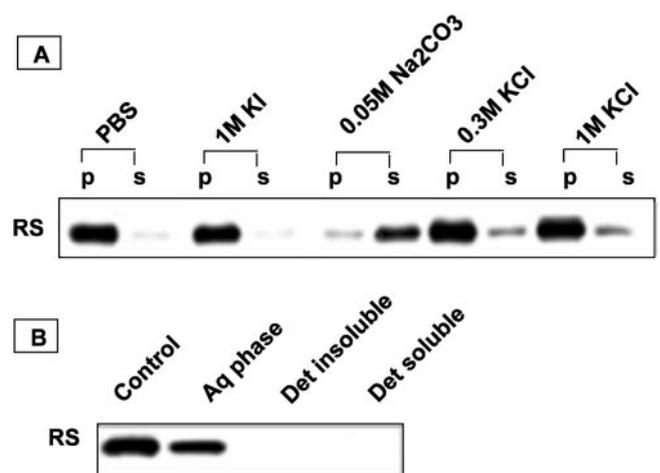
with 0.3 M or 1.0 M KCl or with 1 M KI, most of the protein was detected in the 100,000g pellets. In control experiments, none of the proteins was solubilized when the membrane fractions were treated with PBS. These data suggest that, on being secreted, the RS protein becomes associated with the membrane.

Membrane association of RS was also demonstrated by cloudy point precipitation with Triton X-114, which has been widely used for the separation of integral membrane proteins from soluble proteins.<sup>19</sup> As shown in Figure 2B, most of the RS from membranes segregated into the aqueous phase on Triton X-114 solubilization and phase separation, which is characteristic of peripheral membrane proteins.

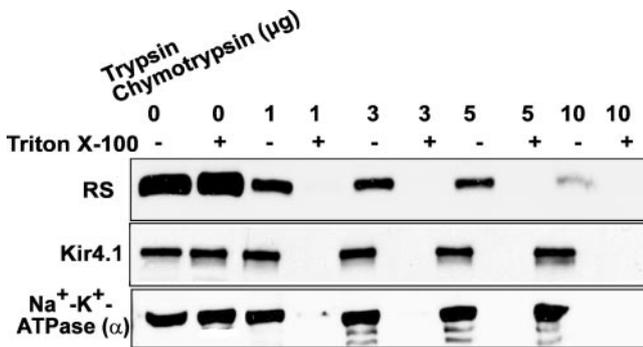
### Membrane Orientation of RS

As a means to verify the membrane orientation of RS, the membrane fractions were subjected to protease (trypsin/chymotrypsin) digestion in the presence or absence of Triton X-100, and the protease resistant membrane fractions recovered in pellets were analyzed for RS by immunoblot analysis (Fig. 3). Protease treatment selectively digests proteins that face the extracellular space. Protein epitopes that reside within the transmembrane domain or on the cytoplasmic side of the plasma membrane are protected from digestion in the absence of detergent. However, treatment of the membrane fractions with detergent before protease digestion disrupts the membrane integrity and allows access of the protein epitopes to the protease.

The integral plasma membrane proteins Na<sup>+</sup>,K<sup>+</sup>-ATPase and inwardly rectifying potassium channel 4.1 (Kir4.1) were used as positive controls to assess the integrity of the membrane vesicles used for establishing RS orientation. The polyclonal antibodies H-300, raised against amino acids 551-850 mapping within the transmembrane and cytosolic regions of Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ 1, and APC-035, raised against C terminus amino acids 356-375 of Kir4.1, detected their respective proteins in membranes that were digested with the protease in the absence of detergent (Fig. 3). This result indicates that the transmembrane domain, protected by the lipid bilayer, and the intracellular C terminus domain were inaccessible to externally added protease and thus remained insensitive to protease di-



**FIGURE 2.** RS is a peripheral membrane protein and is released from the membrane after alkaline extraction or detergent extraction. (A) Alkaline and high salt extraction of membrane fractions isolated from mouse retina. The pellets (p) and supernatants (s) were analyzed for RS by immunoblot analysis. (B) Triton X-114 extraction of membranes and phase separation. Equal volumes of aqueous phase (Aq phase), detergent insoluble (det insoluble), and detergent soluble (det soluble) fractions were analyzed for RS as in (A).



**FIGURE 3.** Membrane orientation of RS, as determined by proteolysis. Membrane fractions were incubated at 0°C with increasing amounts of trypsin/chymotrypsin, with or without 1% Triton X-100. Proteins were analyzed by Western blot analysis. The primary antibodies used were directed against the N terminus epitope of RS, a cytosolic epitope of Kir4.1 and transmembrane and cytosolic epitopes of Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ 1. The apparent molecular weight of the proteins: Na<sup>+</sup>,K<sup>+</sup>-ATPase, 113 kDa; Kir4.1 tetramer, ~160 kDa; and RS monomer, 26 kDa.

gestion in the absence of the detergent. However, both Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ 1 and Kir4.1 disappeared on immunoblots when membrane integrity was first compromised by Triton X-100, so as to allow access of the protease to the protein epitopes. These results thus confirm that most of the membrane vesicles used in this study were in the right-side-out (extracellular space-side-out) orientation.

The majority of RS (~75%–80%) was sensitive to protease in the absence of detergent and was digested at the lowest concentration of the protease (1  $\mu$ g, Fig. 3). This result indicates that this pool of RS is located on the external surface of the plasma membrane and therefore is readily accessible to protease. Of interest, 20% to 25% of RS remained resistant to protease in the absence of detergent, even at higher concentrations of the protease. This pool of RS is not inherently protease resistant, because it was digested when membrane integrity was disrupted with detergent before protease treatment. Since RS lacks a transmembrane domain, this result is consistent with the possibility that the protease-resistant RS is associated with the cytoplasmic side of the plasma membrane. All these data taken together suggest that a major pool of RS is on the outer surface of the plasma membrane, whereas 20% to 25% of RS remains bound to the intracellular surface.

### RS Interaction with Membrane Phospholipids

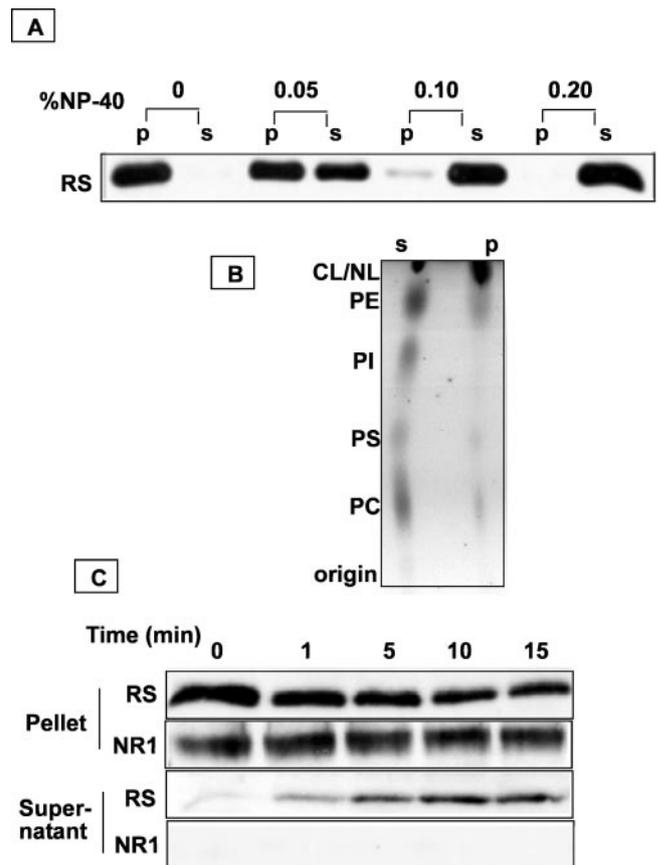
Membrane extractions were performed in the presence of various concentrations of the nonionic detergent NP-40. With centrifugation, RS partitioned totally into the soluble (supernatant) phase at detergent concentrations as low as 0.1% (Fig. 4A). Glycerophospholipids were also preferentially solubilized at these low concentrations of the detergent. Qualitative analysis of the lipids in the supernatant fractions by thin layer chromatography revealed almost total partitioning of the glycerophospholipids (phosphatidylcholine [PC]; phosphatidylserine [PS]; phosphatidylinositol [PI]; phosphatidylethanolamine [PE]) into the soluble phase (Fig. 4B). This implies that RS is extracted together with the glycerophospholipids after NP-40 treatment. Cholesterol was not resolved in this solvent system. However, it is known that at such low concentrations of detergent most of the cholesterol and sphingomyelin remain in the membrane.<sup>18</sup> Thus, RS is not likely to be associated with membrane cholesterol or sphingomyelin, as discussed in the following sections.

The finding that RS is present in the membrane fraction and can be extracted together with the glycerophospholipids after

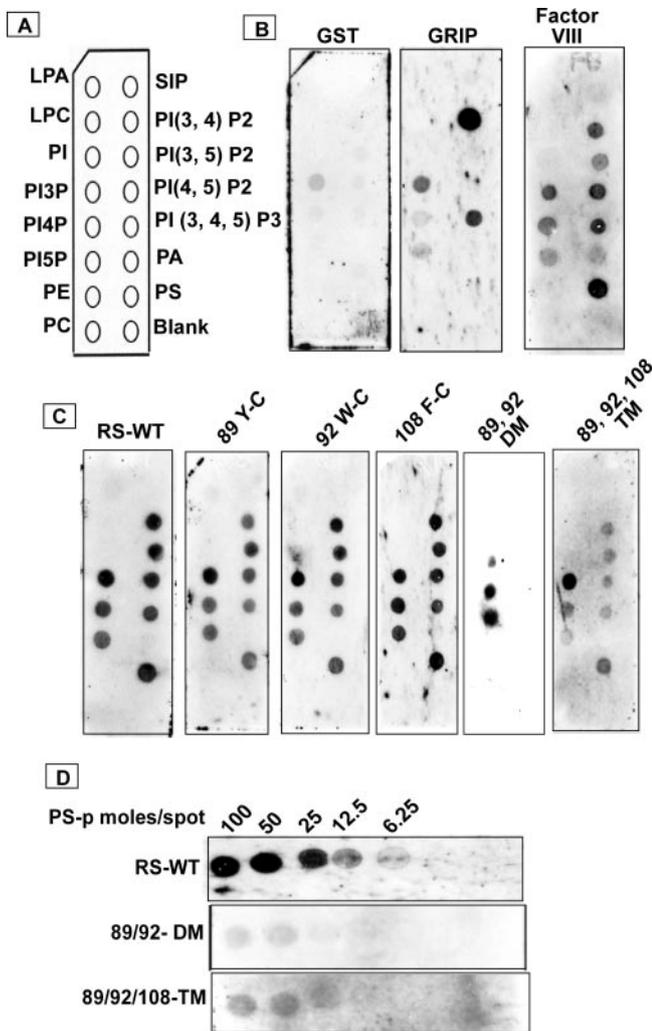
NP-40 treatment suggested that RS may be bound to membrane lipids. Furthermore, the discoidin domains of blood coagulation factors V and VIII were shown to bind phospholipids. Hence, to ascertain whether RS is directly associated with membrane phospholipids, the retinal membrane fractions were subjected to phospholipase C treatment. RS was released from the membrane after PLC treatment (Fig. 4C) which preferentially hydrolyzes the phosphate bond on PC, PS, and PE. RS was increased in the supernatant with a concomitant decrease in the membrane pellet fractions in a time-dependent manner. The specificity of this reaction was confirmed by probing the membrane and pellet fractions with an antibody to non-NMDA receptor subunit 1 (NR1). NR1, a transmembrane protein embedded in the membrane, cannot be easily removed by PLC treatment, and consistent with this, PLC failed to release NR1 from the membrane into the supernatant (Fig. 4C). The above findings confirm that RS is associated with phospholipids on the membrane surface.

### RS Binding to Negatively Charged Phospholipids in Protein–Lipid Overlay Assays

To assess the lipid-binding properties of RS directly, we assayed full-length RS discoidin domain (RS-WT) fusion proteins



**FIGURE 4.** RS was extracted together with the glycerophospholipids after NP-40 treatment. (A) Retinal membrane preparations were incubated with NP-40 at the indicated concentrations. After the incubation, the insoluble pellet (p) and soluble supernatant (s) fractions were isolated and analyzed for RS by immunoblot analysis. (B) Retinal membranes were incubated in the presence of 0.1% NP-40 and after low-speed centrifugation, equal volumes of the resultant pellets (p) and supernatants (s) were analyzed by TLC for lipid profile. (C) Phospholipase C (PLC) treatment released RS from membranes. Retinal membranes were incubated in PBS (80  $\mu$ g protein in 100  $\mu$ L) in the presence of 1 IU of PLC for various times at 25°C; and, after incubation and low-speed centrifugation, equal volumes of the resultant pellet and supernatant fractions were analyzed for RS or non-NMDA receptor subunit 1 (NR1). CL, Cardiolipin; NL, Neutral lipids.



**FIGURE 5.** Protein-lipid overlay assays. **(A)** Schematic diagram of the nitrocellulose membranes with immobilized phospholipids in 100-picomole spots (PIP Strip). **(B)** PIP strips were incubated with the purified GST fusion proteins at 200 ng/mL. GST (negative control) and GRIP and factor VIII (positive controls). The membranes were washed, and lipid-bound GST fusion proteins were detected with monoclonal antibodies against GST or factor VIII. **(C)** PIP strips were incubated with purified wild-type or mutant versions of GST-RS discoidin domain recombinant proteins (200 ng/mL) and the lipid-bound protein was detected with anti-RS antibody. **(D)** Membrane lipid arrays containing serial dilutions of phosphatidylserine (PS) were incubated with purified wild-type or mutant versions of GST-RS discoidin domain recombinant proteins (200 ng/mL), and the lipid-bound protein was detected. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PI, phosphatidylinositol; PI3P, PtdIns(3)P; PI4P, PtdIns(4)P; PI5P, PtdIns(5)P; PI(3,4)P<sub>2</sub>, PtdIns(3,4)P<sub>2</sub>; PI(3,5)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub>; PI(4,5)P<sub>2</sub>, PtdIns(4,5)P<sub>2</sub>; PI(3,4,5)P<sub>3</sub>, PtdIns(3,4,5)P<sub>3</sub>; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SIP, sphingosine 1-phosphate.

for binding to phospholipids in a lipid blot (Fig. 5). The controls gave results consistent with their known binding specificities, and GST alone did not bind lipids (Fig. 5B). The positive control, GST-GRIP (Multi PIP Grip; Echelon Biosciences) bound primarily PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>. Factor VIII bound phosphatidylserine (PS) preferentially compared with other negatively charged phospholipids, which is consistent with the selectivity of the C2 discoidin domain for PS.<sup>21</sup> The recombinant RS wild-type discoidin domain (RS-WT) displayed moderate- to high-affinity binding to negatively charged phos-

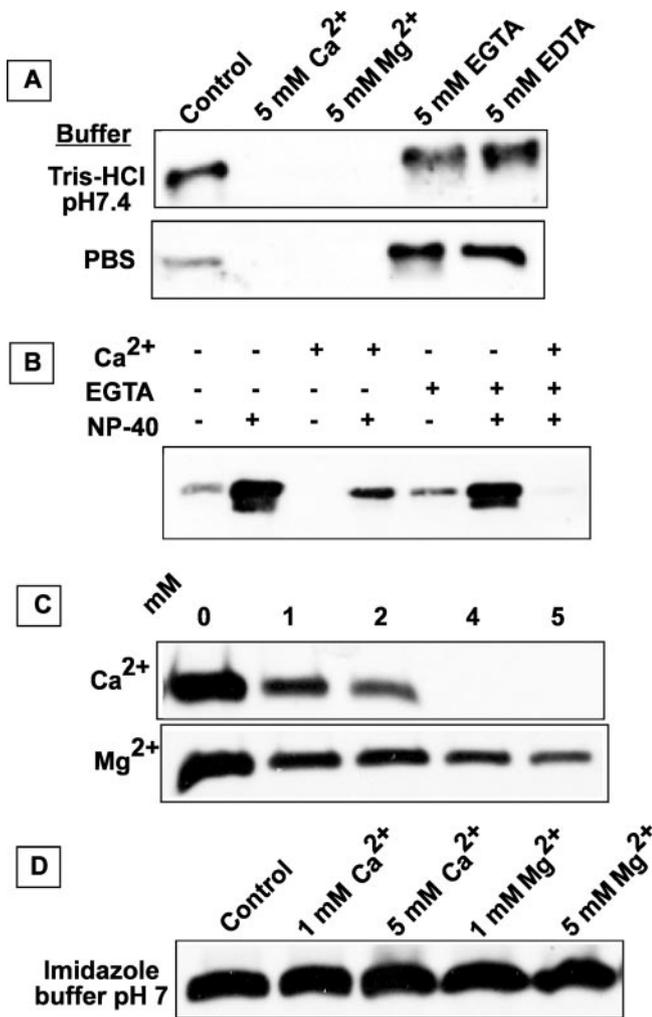
pholipids and bound robustly to PtdIns(3)P, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub>, PtdIns(4,5)P<sub>2</sub>, and PS (Fig. 5C). RS-WT also displayed moderate binding to PtdIns(4)P, PtdIns(5)P, and PtdIns(3,4,5)P<sub>3</sub>. RS-WT did not bind to phosphatidylethanolamine (PE), phosphatidylcholine (PC), and other lysophospholipids (Fig. 5C). These results reflect the general affinity of RS for negatively charged membrane phospholipids. Such RS-lipid interactions have also been suggested by a 3D structure model of RS that has been generated by sequence alignment of RS with the discoidin domain sequences and the crystal structures of factors V and VIII.<sup>22</sup>

Site-directed mutagenesis was performed to determine whether RS has specific affinity for one of the negatively charged phospholipids, rather than a general affinity for negatively charged membrane surfaces. The molecular model that proposed RS-lipid interactions<sup>22</sup> predicted that three aromatic amino acids—Tyr-89 (89Y), Trp-92 (92W), Phe-108 (108F)—form the membrane lipid anchoring site, and these amino acids are mutated to Cys (C) in some families with XLR disease (<http://www.dmd.nl/rs/> Leiden Muscular Dystrophy Pages, Leiden University Medical Center, Leiden, The Netherlands). We found that replacing either Tyr-89 or Trp-92 with Cys reduced the affinity of RS-DD binding to PS by 30% to 50% (Fig. 5C). Both the mutants bound negatively charged phosphoinositides with various affinities. Phe-108 (Cys) mutant showed slightly enhanced binding to all anionic phospholipids. When Cys was substituted for both of the aromatic residues Tyr-89/Trp-92 (double mutant, DM) simultaneously, RS totally lost its affinity for binding to PS but showed enhanced binding to PtdIns(4)P and PtdIns(5)P. Substitution of Cys at all the three aromatic amino acid residues (Tyr-89/Trp-92/Phe-108; triple mutant, TM) decreased the affinity of RS for binding to PS. However, the triple mutant retained its affinity for binding to PtdIns(3)P. The higher affinity of RS for PS was further shown by repeating the binding assay on membranes that contained serial dilutions of the PS (Fig. 5D). Simultaneous mutations at two or three critical amino acid residues (Tyr-89, Trp-92, and Phe-108) abolished RS binding to PS. These results show the preferential binding of RS to PS and the importance of the conserved aromatic residues Tyr-89 and Trp-92 in anchoring RS to membrane phospholipids.

### Influence of Ionic Milieu on RS-Membrane Association

The influence of ionic environment on the association of RS with anionic phospholipids was analyzed by examining the effect of divalent cations (Ca<sup>2+</sup>/Mg<sup>2+</sup>) and chelating agents (EDTA/EGTA) on the release of RS from freshly isolated retinal cell membranes into the incubation medium. RS in the medium was analyzed by Western blot (Fig. 6). Incubation of membranes in Tris-HCl (pH 7.4; physiological strength: 160 mM) buffer alone yielded increased release of RS from membranes compared to PBS (Fig. 6A). Inclusion of either Ca<sup>2+</sup> or Mg<sup>2+</sup> (5 mM) in the buffer prevented the release of RS from the membranes, whereas similar concentrations of EGTA or EDTA resulted in the highest release of RS (Fig. 6A). The presence of protease inhibitors in the incubation media excluded the contribution of proteases to the release of RS. Most important, extraction of RS by NP-40 was prevented by elevating the Ca<sup>2+</sup> (5 mM) concentration in the medium (Fig. 6B). In this case, equimolar concentrations of EGTA did not reverse the effect. Probably a higher concentration of chelating agent is needed.

To determine more about the divalent cation-mediated association of RS with the membranes, we incubated the membranes in NP-40-containing media with increasing concentrations of either Mg<sup>2+</sup> or Ca<sup>2+</sup> and the incubation media were



**FIGURE 6.** Divalent cations Ca<sup>2+</sup> and Mg<sup>2+</sup> inhibit the release of RS from membranes. (A) The effects of divalent cations and chelating agents on the release of RS from freshly isolated retinal cell membranes incubated either in PBS or 160 mM Tris-HCl (pH 7.4). RS was detected by immunoblot analysis. In (B–D) membranes were extracted with NP-40 after preincubation with EGTA, Mg<sup>2+</sup>, or Ca<sup>2+</sup> (5 mM). The buffers used were (B) PBS, (C) sucrose 320 mM-HEPES 10 mM, and (D) 120 mM KCl, and 20 mM imidazole (all pH 7.4).

analyzed by Western blot for RS. The results show a progressive decrease in the release of RS from the membranes (Fig. 6C). At similar concentrations Ca<sup>2+</sup> was a more effective inhibitor than was Mg<sup>2+</sup>. In previous studies, the effects of Ca<sup>2+</sup> were studied in medium that contained imidazole.<sup>18</sup> However, the inhibitory effect of divalent cations was abolished completely when the retinal cell membranes in imidazole media (20 mM) were extracted with NP-40 (Fig. 6D). Imidazole is an effective chelator of the divalent cations and facilitated the detergent-mediated release of RS from the membrane. We conclude from these results that divalent cations contribute to the association of RS with membrane phospholipids.

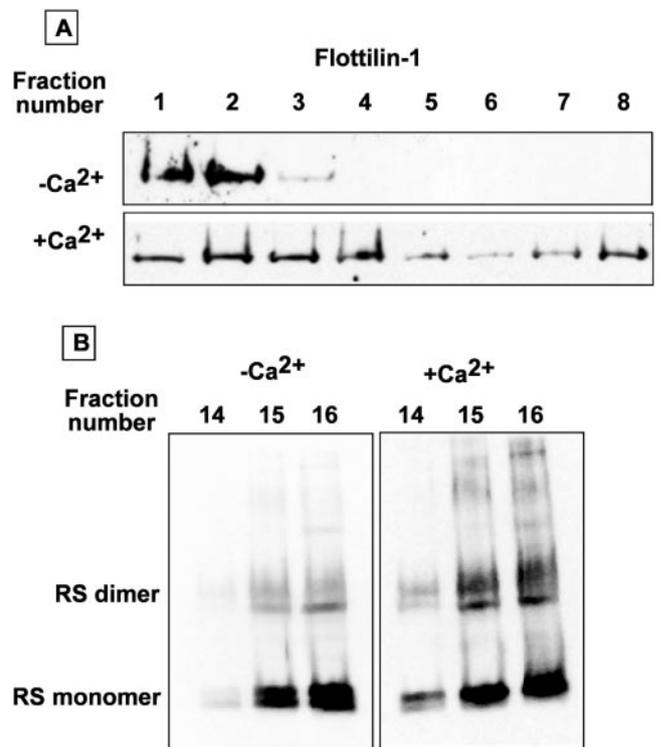
**Ca<sup>2+</sup> Dependent Changes in Membrane**

We investigated whether the inability of detergent to release RS from the membrane in the presence of Ca<sup>2+</sup> might result from a Ca<sup>2+</sup> induced membrane restructuring and redistribution of RS in the membrane. Membrane changes were assessed by analyzing the distribution of lipid rafts. Mouse retinas were solubilized in Triton X-100 both in the absence and presence of

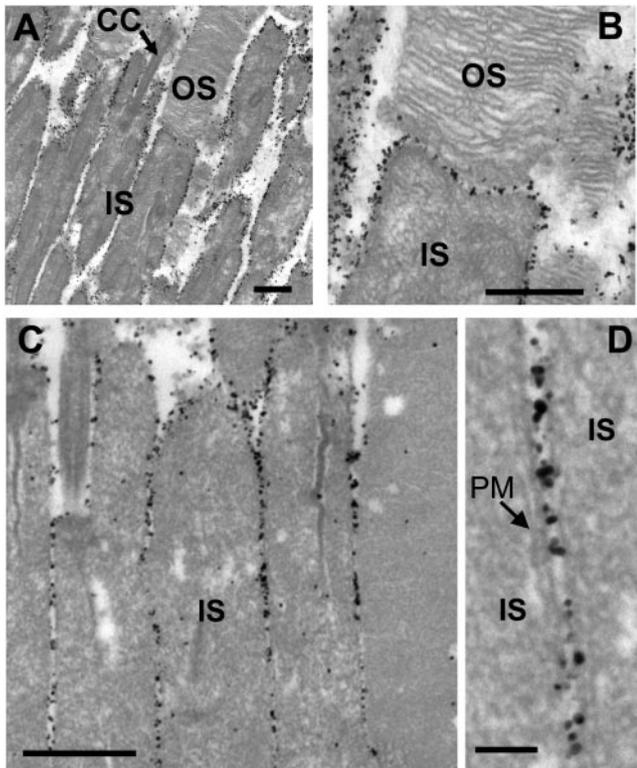
5 mM Ca<sup>2+</sup>, and lipid rafts were prepared by discontinuous iodixanol density gradient fractionation. In the absence of Ca<sup>2+</sup>, the raft membrane marker flotillin-1 was found primarily in lighter iodixanol gradient density fractions 1 to 4 (Fig. 7A). Proteins associated with cholesterol and sphingomyelin are known to sediment in these lighter density fractions.<sup>25</sup> Preparation of membrane rafts in the presence of Ca<sup>2+</sup> gave recovery of flotillin-1 across a broader density range of bands 1 to 8, indicating a structural rearrangement in the membrane and redistribution of flotillin-1 (Fig. 7A). However, RS was distributed only in the high-density fractions 14 to 16 (Fig. 7B), consistent with lack of association of RS with cholesterol or sphingomyelin. In contrast to flotillin 1, the presence of Ca<sup>2+</sup> did not shift the RS gradient pattern, as RS was once again recovered in fractions 14 to 16 of the membrane raft preparation. The slight increase in the intensity of fraction 14 (Fig. 7B) in the presence of Ca<sup>2+</sup> was deemed not significant. Hence, although a Ca<sup>2+</sup>-dependent membrane restructuring caused a redistribution of flotillin-1, this was not accompanied by any apparent Ca<sup>2+</sup>-dependent change in RS distribution in the membrane.

**RS Immunolocalization in Photoreceptors**

Electron microscopy with immunogold labeling showed that RS was densely localized on the plasma membrane of the photoreceptor inner segment (Figs. 8A, 8B), consistent with biochemical observations that RS is mainly associated with membrane fractions enriched in inner segments. RS immunolabeling was concentrated at the margin between tightly packed inner segments (Fig. 8C). At higher magnification, it is apparent that the immunogold labeling is associated with the



**FIGURE 7.** Calcium-dependent changes in the membrane. (A) The effect of Ca<sup>2+</sup> on membrane restructuring was assessed by using the distribution of the lipid raft marker flotillin 1 in iodixanol discontinuous density gradient fractionations 1 to 8 (1, light). (B) The effect of Ca<sup>2+</sup> on the distribution of RS in the membrane fractions 14 to 16. In both cases, mouse retinas were solubilized in Triton X-100 in the absence or presence of 5 mM Ca<sup>2+</sup>.



**FIGURE 8.** Localization of RS on the inner segment plasma membrane. (A, B) Anti-RS antibody in immunogold EM micrographs with WT mice showed labeling of the surface of the photoreceptor inner segment plasma membrane but not the connecting cilium, the outer segment, or extracellular space. (C) Dense labeling was seen between adjacent membrane surfaces of tightly packed inner segments and appeared to be restricted to the outer leaflet of the plasma membrane (D). CC, connecting cilium; OS, outer segment; IS, inner segment; PM, plasma membrane. Scale bars: (A–C) 500 nm; (D) 100 nm.

extracellular surface, or outer membrane leaflet, of the inner segment plasma membrane. Some of the immunogold label in Figure 8D appears to show examples of linkage of two adjacent inner segments through RS molecules. Little or no immunoreactivity was present on connecting cilia, outer segments, or in the extracellular space (Figs. 8A, 8B). In addition, none was seen on the retinal pigment epithelium (data not shown).

Electron microscopy also showed inner segment membrane changes in the absence of RS expression in *RS1*<sup>-/-</sup> mice. Whereas the photoreceptor inner and outer segments in wild-type retina were closely packed and well organized (Fig. 9A), in the knockout *RS1*<sup>-/-</sup> mouse retina the inner segment membranes were irregular, and the morphology of the inner segments themselves was disrupted (Fig. 9B), with large gaps between adjacent cells (Fig. 9B). The shape and structure of mitochondria were markedly altered, and the ground substance of the cytoplasm appeared coarse compared with the wild-type retina.

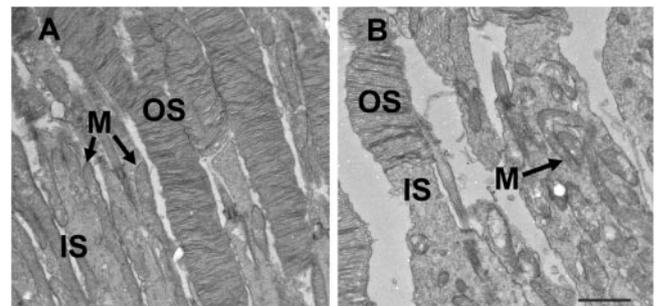
## DISCUSSION

The biochemical and ultrastructural characteristics of RS in intact retina provide evidence that RS is a peripheral membrane protein bound by ionic forces to the outer leaflet of the photoreceptor inner segment plasma membrane. Electron microscopy of immunogold-labeled retinal sections demonstrated clustering of gold particles on the outer surface of the inner segment plasma membrane and the absence of binding to the

outer segment membrane and connecting cilium. Our study also supports the existence of an intracellular RS pool. It is not clear what, if any, functional role this intracellular membrane-bound RS plays or whether it merely represents the cargo that is ready to be secreted by the cell.

A correlation between the detergent extraction properties of RS and glycerophospholipids provided the initial evidence that RS is anchored to the membrane through an association with glycerophospholipids. Further work demonstrated a general affinity of RS for the negatively charged glycerophospholipids on the membrane surface. Our data suggest that the interactions with negatively charged glycerophospholipids are critical for RS binding to membranes and that RS exhibits a preference for binding to certain lipid moieties, such as phosphatidylserine. The affinity of RS for membrane surface was also influenced by divalent cations. The lipid-binding properties of RS as well as its sensitivity to divalent cations are consistent with charge interactions that are important structural and functional features of proteins. Divalent cations mediate an interaction between a protein and its ligand and also influence the binding of proteins to phospholipids. Many examples of proteins with Ca<sup>2+</sup>-dependent phospholipid binding characteristics exist. Annexin II is present in both extracellular and intracellular compartments and binds phospholipids in a Ca<sup>2+</sup> dependent manner to bring about a new configuration of the membrane-bound components.<sup>24</sup> Ca<sup>2+</sup>-dependent phospholipid binding to the C2A and C2B domains of synaptotagmin 1 triggers neurotransmitter release.<sup>25</sup> Ca<sup>2+</sup> has also been shown to induce restructuring and sugar-binding affinity of discoidin 1, an adhesive protein from *Dictyostelium discoideum*.<sup>26</sup> Although our in vitro data revealed that Ca<sup>2+</sup> caused membrane remodeling, it did not alter the distribution of RS on the membrane.

What little we know about the molecular interactions of RS has been extrapolated from knowledge about the conserved 157 amino acid discoidin domain sequence that comprises approximately 75% of the mature RS protein. Several families of extracellular and transmembrane proteins involved in cell adhesion, cell-cell interaction, and cell signaling, including the blood coagulation factors V and VIII and the discoidin domain receptors 1 and 2 (DDR1 and DDR2), display this conserved sequence in single or multiple copies.<sup>14</sup> RS shares 37.2% sequence homology with factors V and VIII. In fact, the molecular basis of the protein defects underlying the XLRS pathology has been predicted from the sequence alignment of RS with the discoidin domain sequences of factors V and VIII and from a 3D model of RS that was generated from factors V and VIII crystal structure data.<sup>27</sup> Phospholipids and collagens serve as ligands for discoidin domains present in blood coagulation



**FIGURE 9.** Photoreceptor inner segment ultrastructure in 2-month-old *RS1*<sup>-/-</sup> and WT mice retinas. Electron micrographs of cross sections through wt (A) and mutant (B) photoreceptor inner and outer segments. Disorganization of the inner segment structure occurred in *RS1*<sup>-/-</sup> mice. CC, connecting cilium; IS, inner segment; m, mitochondria. Scale bar, 500 nm.

factors and discoidin domain receptors, respectively.<sup>14</sup> Our finding that RS associates with phospholipids is in agreement with the 3D model calculated for RS, which predicted a possible role of phospholipids in RS interactions.<sup>22</sup> Consistent with this model, mutagenesis of the two conserved aromatic amino acids Tyr-89 and Trp-92, which had been predicted to act as membrane interaction spikes, affected the binding of RS to phosphatidylserine. Together, these results suggest that negatively charged anionic lipids are critical for RS binding to plasma membrane and RS exhibits a preferential affinity for phosphatidylserine.

Two types of functional interactions between RS and membranes can be envisioned. RS bound to the cell surface via lipid or ionic interactions may provide the molecular architecture necessary to stabilize membranes. Alternatively, RS may mediate the association of the extracellular matrix with the surface of photoreceptor and other retinal cells to promote cell adhesion and thereby stabilize the cellular architecture of the highly structured retinal tissue. In the homo-octameric form,<sup>11</sup> RS that is bound to cell membrane through phospholipids would have sites open to interact with other extracellular proteins such as collagens and with carbohydrates. Although there is no current evidence of type I collagen in the retina, we found that full-length recombinant RS could bind to type I collagen *in vitro* (results not shown). However, our immunoprecipitation experiments (results not shown) gave no evidence that RS interacts with collagen types II, III, and XVII, which are present in the retina.<sup>28,29</sup> It may be difficult to identify RS complexes by coimmunoprecipitation from detergent cell lysates, because these complexes represent only a minor fraction of total cellular proteins and therefore are difficult to target with antibodies. Although a recent study demonstrated the association of RS with extracellular  $\beta$ 2 laminin and intracellular  $\alpha$ B crystallin, no direct interaction between RS and the putative interacting protein was established.<sup>30</sup>

Immunoelectron microscopy revealed extensive accumulation of RS on membrane surfaces and indicated binding of RS to the outer leaflet of the photoreceptor inner segment plasma membrane. In addition, loss of RS expression in *RS1*<sup>-/-</sup> mice altered the normal morphology of the inner segment membranes. This suggests that RS may act locally to maintain the photoreceptor inner segment stability and architecture, and that it is not secreted only for export to the inner retina as previously proposed.<sup>12</sup> Most important, the high affinity of RS for membrane surfaces would restrict its diffusion into the extracellular matrix and may be the reason that, despite the close proximity of the photoreceptor outer and inner segments, we found no evidence by electron microscopy that RS is present on connecting cilium and outer segment membranes. In summary, we propose that disruption of the inner segment architecture due to the loss of RS on inner segments may be the basic mechanism that underlies the displacement and disorganization of photoreceptors that we have seen in the *RS1*<sup>-/-</sup> mice retinas.<sup>6</sup> RS bound by phospholipids on the membrane surface can participate in cell-matrix and cell-cell interactions, which will influence cytoskeletal organization. Other adhesion molecules have been shown to integrate signaling events between the extracellular matrix and the intracellular cytoskeleton.<sup>31,32</sup> Future studies are needed to address the molecular mechanisms and the role of RS in such events.

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## E R R A T U M

**Erratum in:** "The Induction and Suppression of the Apoptotic Response of HSV-1 in Human Corneal Epithelial Cells" by Miles et al. (*Invest Ophthalmol Vis Sci.* 2007;48:789-796).

The inhibitor for caspase 9 should be listed as Z-LEHD. In the **Materials and Methods** section, under the heading "Caspase Inhibition Assays," the first sentence should read, "Caspases 8 and 9 were inhibited by the amino acid sequences Z-IETD and Z-LEHD, respectively."