Disulfide-mediated Oligomerization of Peripherin/Rds and Rom-1 in Photoreceptor Disk Membranes

IMPLICATIONS FOR PHOTORECEPTOR OUTER SEGMENT MORPHOGENESIS AND DEGENERATION*

(Received for publication, September 15, 1999, and in revised form, December 9, 1999)

Christopher J. R. Loewen‡ and Robert S. Molday§

From the Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Peripherin/Rds is a tetraspanning membrane protein that has been implicated in photoreceptor outer segment morphogenesis and inherited retinal degenerative diseases. Together with the structurally related protein, Rom-1, it forms a complex along the rims of rod and cone disc membranes. We have compared the oligomeric structure of these proteins from nonreduced and dithiothreitol reduced membranes by velocity sedimentation, SDS-gel electrophoresis, immunoaffinity chromatography, and chemical cross-linking. Under reducing conditions, peripherin/Rds and Rom-1 exist as homomeric and heteromeric core complexes devoid of intermolecular disulfide bonds. Under nonreducing conditions core complexes associated through intermolecular disulfide bonds to form oligomers. One intermediate-size oligomer contained monomers and disulfide-linked dimers of peripherin/Rds and Rom-1, while larger oligomers consisted only of disulfide-linked peripherin/Rds dimers when analyzed on nonreducing SDS gels. Consistent with this result, disc membranes contained twice as much peripherin/Rds as Rom-1. Peripherin/Rds individually expressed in COS-1 cells also formed disulfide-linked oligomers bridged through Cys-150 residues, whereas Rom-1 showed little tendency to form oligomers. These results indicate that peripherin/Rds and Rom-1 associate noncovalently to form multisubunit core complexes. Peripherin/Rds containing core complexes interact through specific intermolecular disulfide bonds to form oligomers which may play a crucial role in photoreceptor disc morphogenesis and retinal degenerative diseases.

Phototransduction takes place in a specialized compartment of the rod and cone photoreceptor cell called the outer segment. This compartment consists of a stack of highly ordered discs surrounded by a plasma membrane. A disc is composed of two closely spaced lamellar membranes that are fully (rods) or partially (cones) circumscribed by a hairpin membrane called the disc rim. This region is generally thought to play an essential role in the morphogenesis and stabilization of the outer segment, although the molecular mechanism and interactions are not yet known (1–4).

Peripherin/Rds and Rom-1 are two membrane proteins that are localized along the rim and incisures of rod and cone disc membranes (5–9). Peripherin/Rds is required for rod and cone outer segment morphogenesis and stabilization since rds mice homozygous for the disrupted peripherin/rds gene fail to develop outer segments and mice heterozygous for this gene defect form highly disorganized structures consisting of whorls of membranes (10–14). Moreover, mutations in the peripherin/rds gene have been linked to a variety of inherited human retinal diseases including autosomal dominant retinitis pigmentosa, macular degeneration, and related pattern dystrophies (15–20). Recent studies with transgenic mice have confirmed that disease-linked mutations in peripherin/rds cause outer segment disorganization and photoreceptor degeneration (21).

Rom-1 plays a more ancillary role in outer segment structure. In the absence of Rom-1, characteristic rod and cone outer segments containing stacks of discs are evident (22). The discs in rod cells, however, tend to be slightly longer than normal and occasionally have a disorganized appearance. Furthermore, to date mutations in the rom-1 gene have been linked only to a digenic form of autosomal dominant retinitis pigmentosa (23, 24). In this case, the disease phenotype is evident only in individuals who inherit a L185P mutation in peripherin/rds along with a rom-1 mutation.

Peripherin/Rds and Rom-1 are similar in size and exhibit many common structural features (25). They both contain four putative membrane spanning segments, an extended cytoplasmic C-terminal domain, a large intradiscal loop of approximately 150 amino acids that joins the third and forth transmembrane segments, and seven highly conserved cysteine residues (6–8, 26). The C terminus of peripherin/Rds has been reported to induce membrane fusion in vitro suggesting a possible role for this domain in the outer segment renewal process (27). The large intradiscal loop of peripherin/Rds, however, contains most disease causing missense mutations and appears to play a crucial role in protein-protein interactions important for disc formation and stabilization (28, 29).

Peripherin/Rds and Rom-1 assemble into a multisubunit protein complex (7–8, 30). Earlier biochemical studies suggest that this complex is a tetramer composed of peripherin/Rds and Rom-1 subunits (31). Intermolecular disulfide bonds have been generally thought to be involved in maintaining the structure of this complex since a significant fraction of peripherin/Rds and Rom-1 migrates as disulfide-linked dimers on nonreducing SDS-polyacrylamide gels (5, 7, 32). More recently, mutagenesis studies have identified a single cysteine residue (Cys-150) in the large intradiscal loop of peripherin/Rds that is responsible...
for the formation of disulfide-linked peripherin/Rds dimers (29).

To further define the role of intermolecular disulfide bonds in the structure of the peripherin/Rds-Rom-1 complex, we have analyzed the oligomeric structure of these proteins from reduced and nonreduced ROS and monkey kidney COS-1 cell membranes by velocity sedimentation, SDS-gel electrophoresis, immunoaffinity chromatography and covalent cross-linking. We report here that peripherin/Rds and Rom-1 associate noncovalently to form a mixture of homomeric and heteromeric core complexes. A significant fraction of peripherin/Rds-containing core complexes further link together through intermolecular disulfide bonds to form higher order oligomers. These studies lead to a new model for the oligomeric structure of peripherin/Rds containing proteins in ROS and provide insight into the possible role of these complexes in outer segment morphogenesis and retinal degeneration.

**EXPERIMENTAL PROCEDURES**

**ROS Preparations and Monoclonal Antibodies—**ROS were isolated from frozen bovine retina by sucrose gradient centrifugation as described previously (33). Monoclonal antibodies to peripherin/Rds (Per2B6) and Rom-1 (Rom1C6) have been reported (5, 8).

**Dimer Reduction Kinetics—**ROS were washed three times under dim red light with 10 volumes of 10 mM Tris-HCl, pH 7.4, and resuspended at a protein concentration of 2 mg/ml in the same buffer. Reduction in the presence or absence of 1% Triton X-100 was initiated at 25 °C by the addition of DTT, cysteine, or glutathione to yield a final concentration of 10 mM. Aliquots were removed at 5-min intervals and added to an equal volume of stop buffer (2% Triton X-100, 120 mM NEM, 0.2 mg/ml PMSF, 10 mM Tris-HCl, pH 7.4). The samples were centrifuged at 90,000 × g for 30 min and the supernatants were subjected to SDS-PAGE under nonreducing conditions for analysis by Western blotting as described below. For kinetic analysis, the decrease in peripherin/Rds or Rom-1 dimer as a function of time was quantified by scanning the ECL exposed film with a laser densitometer. Kinetics were fitted to a single exponential decay using Sigma Plot (Jandel Scientific).

**DTT and NEM Treatment of ROS and Immunopurification of the Peripherin/Rds-Rom-1 Complex—**ROS were washed three times in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4) and the final pellet was resuspended at a protein concentration of 2 mg/ml in PBS in the presence or absence of 10 mM DTT. After 90 min at 25 °C, ROS samples were solubilized by the dropwise addition of an equal volume of ice-cold solubilization buffer (2% Triton X-100 and 0.2 mg/ml PMSF in PBS) containing 100 mM NEM to obtain a protein concentration of 1 mg/ml. The solution was centrifuged at 90,000 × g for 30 min to remove any insoluble material and the supernatant (solubilized ROS) was used either directly for velocity sedimentation measurements and cross-linking studies or for purification of peripherin/Rds and Rom-1. Peripherin/Rds-Rom-1 complex was typically purified from Triton X-100-solubilized ROS on a Per2B6-Sepharose immunoaffinity matrix as described previously (33). Briefly, 200 μl of solubilized ROS were incubated with 50 μl of matrix for 1 h at 4 °C in Millipore Ultrafree MC 0.45-μm filter unit. The matrix was then washed three times with 0.4 ml of solubilization buffer by low speed centrifugation to remove unbound protein, and the bound peripherin/Rds-Rom-1 complex was eluted with 200 μl of solubilization buffer containing 0.1 mg/ml of the 2B6 competing peptide (DAGQPAPAAG).

**Heterologous COS-1 Cell Expression—**pcPER (wild-type peripherin/ rds), pC551T5 (mutant peripherin/Rds), and pcROM (wild- type Rom1) plasmids used for COS-1 cell transfections have been previously described (29, 30). For heterologous expression, COS-1 cells (~6 × 10⁵ cells/100-mm dish) were transfected with 30 μg of plasmid using calcium chloride and harvested 72 h post-transfection as described (30). The cells were washed twice with PBS and incubated with 270 μl of PBS containing 0.1 mg/ml PMSF and either 10 mM DTT or 40 mM NEM. After 90 min at 25 °C, the cells were solubilized by the addition of 30 μl of 10% Triton X-100 and incubated for 20 min on ice. The solution was centrifuged at 90,000 × g for 10 min and the supernatant was collected and maintained on ice until use.

1 The abbreviations used are: ROS, rod outer segments; DTT, dithiothreitol; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

**Velocity Sedimentation—**Triton X-100-solubilized protein from DTT- or NEM-treated ROS or COS-1 cell membranes or immunoaffinity-purified peripherin/Rds-Rom-1 was applied to 5–20% (w/w) 2-ml sucrose gradients prepared in PBS and containing 0.1% Triton X-100. Routinely, 1 ml DTT was included in the gradients for DTT-treated samples. After centrifugation for 12 h at 50,000 rpm in a Beckman TLS-55 rotor at 4 °C, the bottom of the centrifuge tube was punctured and four-drop fractions were collected by gravity flow. Fractions were incubated with 40 mM NEM for 30 min at 25 °C to block free sulfhydryl groups and neutralize any remaining DTT. The samples were then added to an equal volume of SDS mixture in the absence (or presence) of reducing agent and analyzed by SDS-PAGE and Western blotting.

**Glutaraldehyde Cross-linking—**Reduced or nonreduced, Triton X-100-solubilized ROS (0.1 mg/ml) or purified peripherin/Rds-Rom-1 complex (4 μg/ml) was treated with 50 mM NEM and subsequently incubated with 0.001% or 0.01% glutaraldehyde for 15–30 min at 37 °C. Samples were added to an equal volume of SDS mixture containing β-mercaptoethanol for analysis by SDS-PAGE.

**Quantification of Peripherin/Rds and Rom-1 in ROS—**Peripherin/Rds and Rom-1 subunits, used as standards for quantitative analysis, were isolated as follows: DTT-treated ROS membranes were solubilized with an equal volume of 1% SDS in PBS containing 100 mM NEM and PMSF. After centrifugation for 30 min at 100,000 × g, the supernatant was diluted 10-fold with PBS containing 2% Triton X-100 and Rom-1 was bound to Rom1C6-Sepharose. After extensive washing in the same buffer, bound protein was eluted with 2% SDS containing PMSF for 15 min at 37 °C. The purity of the proteins was confirmed by SDS-PAGE and the protein concentration was determined by the method of Kaplan and Pedersen assay (34). Under these conditions, the purified preparation of the peripherin/Rds subunit lacked Rom-1 and purified Rom-1 subunit was free of peripherin/Rds as determined by Western blotting. The amount of peripherin/Rds and Rom-1 in bovine ROS could not be directly determined from Western blots of ROS since rhodopsin is known to block the electrotransfer of peripherin/Rds and to a lesser extent Rom-1. As a result, the peripherin/Rds complex from nonreduced, Triton X-100-solubilized ROS was quantitatively bound to Per2B6-Sepharose columns and subsequent washing above such that peripherin/Rds was detected in the unbound fraction. After the matrix was thoroughly washed, the bound peripherin/Rds complex was quantitatively eluted from the column with 2% SDS. The bound and unbound fractions, along with the peripherin/Rds and Rom-1 standards, were analyzed by SDS-PAGE and Western blotting under reducing conditions. The amount of peripherin/Rds and Rom-1 was determined from laser densitometry of the ECL signal from Western blots. Values interpolated from standard curves were reported as an average of 3 or more experiments and correlated with the protein content in ROS (34).

**Subunit Composition of Disulfide-linked and Glutaraldehyde Cross-linked Dimers—**To determine the composition of disulfide-linked species, nonreduced ROS were solubilized in an equal volume of denaturing buffer consisting of 1% SDS, 100 mM NEM and PMSF in PBS. The solution was then diluted 10-fold with PBS containing 2% Triton X-100 and PMSF and the peripherin/Rds and Rom-1 containing complexes were selectively adsorbed to either Per2B6-Sepharose or Rom1C6-Sepharose as described above. Bound protein was eluted with 2% SDS in PBS and analyzed under nonreducing conditions by SDS-PAGE and Western blotting.

**RESULTS**

**Reduction of Disulfide-linked Peripherin/Rds Dimers in ROS Membranes—**Previous studies have shown that a substantial portion of peripherin/Rds and Rom-1 from ROS migrates as disulfide-linked dimers on nonreducing SDS-poly-
acrylamide gels (5, 6, 7, 26). To determine if the intermolecular disulfide bond responsible for these dimers can be reduced within the membrane environment, ROS were treated with 10 mM DTT for various times and the disappearance of peripherin/Rds dimer was monitored on Western blots of nonreducing SDS gels. As shown in Fig. 1, A and B, peripherin/Rds-containing dimer was exponentially reduced to monomer by DTT with a half-time of 9.8 ± 0.7 min. A similar rate of reduction was observed for Triton X-100-solubilized peripherin/Rds and for membrane bound and solubilized Rom-1 (data not shown). In contrast, glutathione was ineffective as a reducing agent even in the presence of Triton X-100, presumably due to either lower reactivity or inaccessibility of this reagent to the intermolecular disulfide bond (Fig. 1B). Cysteine, on the other hand, was able to reduce detergent-solubilized peripherin/Rds, but at a slower rate than that observed for DTT (data not shown).

**Velocity Sedimentation Analysis of Peripherin/Rds and Rom-1 from DTT Reduced and Nonreduced ROS—**A two-dimensional separation technique was devised to assess the contribution of intermolecular disulfide bonds to the oligomeric structure of the peripherin/Rds-Rom-1 complex from ROS membranes. In the first dimension, velocity sedimentation was used to resolve oligomeric forms of Triton X-100-solubilized peripherin/Rds-Rom-1 complexes from nonreduced (−DTT) and reduced (+DTT) membranes. In the second dimension, fractions from the velocity sedimentation run were treated with NEM to block free sulfhydryl groups and subjected to SDS-PAGE under nonreducing conditions for the detection of disulfide-linked dimers by Western blotting.

Fig. 2, A and B (left panels), shows the two-dimensional analysis of immunoaffinity purified peripherin/Rds-Rom-1 complex from reduced (+DTT) ROS membranes. Both peripherin/Rds and Rom-1 co-sedimented as a single species (fractions 9–12) with a sedimentation coefficient of 5.1, a value that has been previously reported to correspond to a peripherin/Rds-Rom-1 tetramer (29, 31). Further analysis by SDS-PAGE under nonreducing conditions indicated that this species lacked disulfide-linked dimers.

Peripherin/Rds containing proteins from nonreduced ROS, treated with NEM to prevent secondary sulfhydryl oxidation, showed a more complex behavior (Fig. 2, A and B, right panels). Three peripherin/Rds containing components and two Rom-1 containing components were resolved by velocity sedimentation. Component a (fractions 9–12) sedimented at the same rate as the DTT reduced complex and like this complex consisted solely of peripherin/Rds and Rom-1 monomers when analyzed on nonreducing SDS gels. Component b (fractions 5–8) sedimented at a faster rate (s20,w = 7.2) characteristic of a larger oligomer. This component contained both monomers and disulfide-linked dimers of peripherin/Rds and Rom-1 proteins when analyzed on nonreducing SDS gels. Peripherin/Rds containing dimers appeared as a single band, whereas Rom-1 containing dimers were resolved into a doublet. The upper band of the Rom-1 doublet comigrated with the peripherin/Rds band and likely corresponds to disulfide-linked peripherin/Rds-Rom-1 heterodimer. The less intense lower band of the Rom-1 doublet lacked peripherin/Rds, and therefore, most likely corresponds to disulfide-linked Rom-1 homodimers. Component c (fractions 1–3) sedimented with a s20,w of >11 characteristic of a higher-order oligomer. Interestingly, this component lacked Rom-1 and consisted exclusively of disulfide-linked peripherin/Rds homodimers. Similar results were obtained when detergent-solubilized ROS were subjected to velocity sedimentation without prior isolation of the peripherin/Rds complexes except that component c was spread more evenly throughout the lower fractions of the gradient indicative of oligomers of various sizes (data not shown).

The relative amounts of peripherin/Rds and Rom-1 in the three components resolved by velocity sedimentation were determined by Western blotting and laser densitometry. Analysis was performed on the fractions subjected to SDS-PAGE under reducing conditions in which peripherin/Rds and Rom-1 migrated as monomers. Approximately, 35% of peripherin/Rds was present in the core complex (component a), 25% in the intermediate oligomer (component b), and 40% in the higher order oligomer (component c). In the case of Rom-1, 56% of Rom-1 was present in component a and 44% in component b.

These results indicate that peripherin/Rds and Rom-1 interact through noncovalent bonds to form core homomeric and heteromeric complexes, presumably tetramers. A significant portion of these complexes interact through intermolecular disulfide bonds to form larger oligomers, a large fraction of which is devoid of Rom-1.

**Velocity Sedimentation Analysis of Peripherin/Rds and Rom-1 Expressed in COS-1 Cells—**Previously, peripherin/Rds and Rom-1 separately expressed in COS-1 cells were shown to self-assemble into a multisubunit complex that sedimented as a tetramer under mildly reducing conditions (29–31). We have now used the two-dimensional separation technique to analyze for the disulfide-linked oligomerization of individually expressed peripherin/Rds and Rom-1. As shown in Fig. 3, A and B (left panels), both peripherin/Rds and Rom-1 from DTT-treated membranes sedimented as a single species as previously shown (30). Further analysis by SDS-PAGE under nonreducing conditions indicated that these complexes lacked disulfide-linked dimers.

The velocity sedimentation profile of peripherin/Rds from nonreduced (−DTT) COS-1 cell membranes showed larger peripherin/Rds oligomers in addition to the core complex (Fig. 3A, right panel). The oligomers consisted exclusively of disulfide-linked peripherin/Rds dimers when analyzed by SDS-PAGE under nonreducing conditions. In contrast, Rom-1 from nonreduced COS-1 cell membranes showed little tendency to form disulfide-linked oligomers, but instead sedimented primarily as the core complex lacking intermolecular disulfide bonds (Fig. 3B, right panel).
Cysteine at position 150 in the large intradiscal loop of peripherin/Rds has been reported to be responsible for disulfide-linked dimerization of peripherin/Rds (29). To further examine the role of this cysteine in oligomerization, the sedimentation behavior and disulfide-linked dimerization of the C150S peripherin/Rds mutant was examined. As shown in Fig. 3C, the C150S mutant from reduced (+DTT) and nonreduced (−DTT) COS-1 cell membranes sedimented as a single core complex devoid of intermolecular disulfide bonds. These studies indicate that Cys-150 mediates disulfide-linked oligomerization of peripherin/Rds core complexes.

**Cross-linking of the Peripherin/Rds-Rom-1 Complex**—Covalent cross-linking was used to further analyze subunit associations and disulfide-linked oligomerization of peripherin/Rds and Rom-1. In these studies, DTT-reduced and nonreduced ROS membranes were solubilized in Triton X-100, purified on a Per2B6-Sepharose matrix, and cross-linked with glutaraldehyde for analysis by SDS-PAGE under reducing conditions. The Western blot in Fig. 4A shows that a substantial portion of peripherin/Rds from reduced membranes was cross-linked to dimers. Only a faint band corresponding to a tetramer was detected when a relatively high glutaraldehyde concentration (0.01%) was used. In contrast, cross-linking of peripherin/Rds from nonreduced, NEM-treated membranes produced a series of high molecular weight cross-linked multimers in addition to monomers and dimers (Fig. 4B). A similar pattern of cross-linking was observed when Western blots were labeled for Rom-1 (data not shown). These results indicate that peripherin/Rds and Rom-1 from DTT-reduced ROS membranes preferentially cross-link into dimers, whereas the protein from nonreduced membranes cross-link into larger multimeric species, a result that is consistent with the presence of large oligomers observed in velocity sedimentation experiments.

**Identification of Disulfide-linked and Glutaraldehyde Cross-linked Hetero- and Homodimers of Peripherin/Rds and Rom-1**—To further investigate the composition of disulfide-linked and glutaraldehyde cross-linked dimers, an immunoaffinity based method was developed to separate covalently linked subunits after SDS denaturation. Immunoaffinity purified complexes from nonreduced ROS membranes were used to determine the composition of disulfide-linked dimers, and detergent-solubilized glutaraldehyde cross-linked sample from DTT reduced ROS membranes was used to analyze the subunit composition of chemically cross-linked dimers. The samples were denatured in SDS to disrupt noncovalent associations, diluted with Triton X-100 to decrease the SDS concentration, and subjected to affinity chromatography to separate peripherin/Rds and Rom-1 containing subunits for analysis by SDS-PAGE and Western blotting.

To test the efficiency of this method, DTT reduced ROS membranes were first denatured with SDS and peripherin/Rds and Rom-1 were isolated on a Per2B6-Sepharose or Rom1C6-Sepharose matrix, respectively. As shown by Western blotting in Fig. 5A (left panel), peripherin/Rds was present only in the bound fraction and Rom-1 was found in the unbound fraction of Per2B6-Sepharose matrix. Similarly, Rom-1 was present only in the bound fraction of Rom1C6-Sepharose matrix (Fig. 5A, right panel) and peripherin/Rds was detected in the unbound fraction.

The subunit composition of disulfide-linked dimers was determined using nonreduced ROS membranes. Fig. 5B shows Western blots of the unbound and bound fractions from Per2B6-Sepharose (left panel) and Rom1C6-Sepharose (right panel). The unbound fraction of Per2B6-Sepharose contained Rom-1 monomer and a smaller amount of disulfide-linked Rom-1 homodimer, but was devoid of peripherin/Rds. The bound fraction contained both peripherin/Rds monomer and disulfide-linked dimer. This dimer migrated more slowly than the Rom-1 disulfide-linked homodimer in the unbound fraction. It most likely represents a mixture of disulfide-linked peripherin/Rds monomers.
Disulfide-mediated Oligomerization of Peripherin/Rds and Rom-1

Fig. 3. Velocity sedimentation and Western blot analysis of heterologously expressed peripherin/Rds and Rom-1 from reduced and nonreduced COS-1 cell membranes. Peripherin/Rds (A), Rom-1 (B), and C150S (C) peripherin/Rds mutant were individually expressed in COS-1 cells. Cells pretreated with DTT (left panels) or without DTT (right panels) were solubilized with Triton X-100 containing NEM and subjected to velocity sedimentation. Fractions were analyzed on nonreducing SDS gels for detection of peripherin/Rds with Per2B6 antibody (A and C) and Rom-1 with Rom1C6 antibody (B).

Peripherin/Rds homodimer and peripherin/Rds-Rom-1 heterodimer that are not resolved in this gel system. An additional Rom-1 containing band was routinely observed above the dimer in the bound fraction. The nature of this species is not known at the present time. The unbound fraction from the Rom1C6-Sepharose matrix contained peripherin/Rds monomer and disulfide-linked homodimer, but no Rom-1. The bound fraction exhibited bands corresponding to Rom-1 monomer and a disulfide-linked dimer. Since peripherin/Rds was also present in the latter, this dimer band appears to contain peripherin/Rds-Rom-1 heterodimer. These results indicate that oligomers are generated through disulfide bridges between peripherin/Rds-peripherin/Rds subunits, peripherin/Rds-Rom-1 subunits and Rom-1-Rom-1 subunits of the core complexes.

Quantitative Analysis of Peripherin/Rds and Rom-1 in ROS—The amount of peripherin/Rds and Rom-1 in ROS was determined by quantifying the amount of each protein present in the bound and unbound fraction of a Per2B6-Sepharose column. Peripherin/Rds was only present in the bound fraction and constituted 2.1% ± 0.3 (n = 6) of the ROS protein by weight. Rom-1 was present in both the bound and unbound fractions and together comprised 1.1% ± 0.2 (n = 6) of the total ROS protein. Approximately 14% of total Rom-1 was detected.
in the unbound fraction. This Rom-1 component lacking peripherin/Rds migrated solely as monomers by SDS-PAGE under nonreducing conditions. The inability of Rom-1 to form disulfide-linked homodimers in the absence of peripherin/Rds is consistent with the behavior of Rom-1 expressed in COS-1 cells.

DISCUSSION

The peripherin/Rds-Rom-1 complex of ROS disc membranes was previously thought to consist of disulfide-linked homodimers of peripherin/Rds and Rom-1 that interact noncovalently to form a heterotetrameric protein (7, 31). This model was based on the findings that 1) a substantial fraction of peripherin/Rds and Rom-1 migrates as disulfide-linked homodimers on nonreducing SDS gels; 2) Rom-1 co-purifies with peripherin/Rds by immunoaffinity chromatography; and 3) detergent-solubilized peripherin/Rds-Rom-1 complex possesses hydrodynamic properties consistent with a tetrameric complex. However, the hydrodynamic experiments were carried out in the presence of DTT and the existence of disulfide-linked dimers under these conditions was not determined.

Velocity sedimentation measurements reported here indicate that this simplified model is not correct. Instead, our results suggest a novel disulfide-mediated oligomerization model as depicted in Fig. 6. Peripherin/Rds and Rom-1 in ROS membranes interact noncovalently to form multisubunit core complexes. A major portion of the peripherin/Rds-containing complexes links together via intermolecular disulfide bonds to form intermediate and higher order oligomers.

The core complex is a mixture of homomeric and heteromeric multisubunit proteins (Fig. 6). Peripherin/Rds-peripherin/Rds

**Fig. 5.** Analysis of subunits involved in disulfide-linked and glutaraldehyde cross-linked dimers. DTT reduced or nonreduced ROS membranes were denatured in 0.5% SDS to disrupt noncovalent interactions. After dilution with Triton X-100, peripherin/Rds containing complexes were isolated on a Per2B6-Sepharose matrix and Rom-1 containing complexes were isolated on a Rom1C6-Sepharose matrix. Equivalent volumes of the initial extract (lane a), the unbound fraction (lane b), and the SDS eluted fraction (lane c) were analyzed on Western blots labeled with Per2B6 or Rom1C6 antibody. Purified peripherin/Rds containing complexes (left panel) and Rom-1 containing complexes (right panel) from DTT-reduced ROS membranes are shown in A and from nonreduced ROS membranes in B. Purified peripherin/Rds containing complex from DTT-reduced ROS membranes cross-linked with 0.01% glutaraldehyde is shown in C.
homomeric and peripherin/Rds-Rom-1 heteromeric core proteins are the most abundant species and readily form disulfide-linked oligomers. Rom-1 homomeric core protein constitutes only about 10% of these complexes and shows little capacity to form disulfide-linked oligomers. The size of the core complex has been previously estimated to be a tetramer by hydrodynamic measurements (31). Subunits of the complex, however, preferentially cross-link into dimers by glutaraldehyde and other cross-linking agents. Previous studies indicate that the large intradiscal loop is involved in noncovalent subunit interactions (28, 29). It is possible that reactive groups in this segment are not accessible for efficient cross-linking of the subunits into tetramers. Alternatively, hydrodynamic measurements may give an overestimation of the size of the complex, possibly due to the inherent problem of accurately determining detergent binding. Further studies are needed to conclusively establish the size of the core complex.

Two classes of peripherin/Rds-containing oligomers are observed by velocity sedimentation under nonreducing conditions. One class designated as component b (Fig. 2) is intermediate in size and contains both peripherin/Rds and Rom-1 subunits. Only some of the subunits in the hetero-oligomers participate in intermolecular disulfide bonds since both monomers and dimers are observed by SDS-PAGE under nonreducing conditions. The size of this oligomer is estimated to be twice the size of the core complex based on the relationship of the sedimentation coefficients to molecular weights (35) and the assumption that both species bind similar amounts of detergent per core complex. The intermolecular disulfide bonds that link the peripherin/Rds containing core complexes together are formed between two peripherin/Rds subunits, one peripherin/Rds and one Rom-1 subunit, and two Rom-1 subunits. The second, higher order class of oligomers is composed exclusively of peripherin/Rds subunits, all of which participate in intermolecular disulfide bonds. The various oligomeric species are depicted in Fig. 6.

Treatment of ROS membranes with DTT results in the complete breakdown of the oligomers into core complexes indicating that the intermolecular disulfide bonds are readily accessible to this reducing agent and essential for oligomerization. Quantitative analysis indicates that peripherin/Rds is present at almost twice the concentration of Rom-1 in ROS, a finding that is consistent with the presence of a significant amount of peripherin/Rds homo-oligomers detected by velocity sedimentation analysis. Earlier studies failed to detect the presence of peripherin/Rds and Rom-1 homotetrameric core complexes in ROS (7, 8). This may be due to the inability to detect smaller amounts of peripherin/Rds and Rom-1 in the presence of large

![Disulfide-mediated Oligomerization Model](http://www.jbc.org/)

**Fig. 6.** Disulfide-mediated oligomerization model for peripherin/Rds and Rom-1. Peripherin/Rds (dark fill) and Rom-1 (white fill) interact noncovalently via their large intradiscal loops to produce homotrameric and heterotrameric core complexes. The Cys-150 of peripherin/Rds and corresponding Cys-153 of Rom-1 located within the large intradiscal loop of these proteins are in their reduced form (SH). A large portion of the peripherin/Rds homotetramers link together through Cys-150 mediated intermolecular disulfide bonds to form intermediate size homo-octamers. These oligomers can further associate to form higher order disulfide-linked homo-oligomers. All subunits in the peripherin/Rds homo-oligomers contain intermolecular disulfide bonds since only disulfide-linked dimers are observed on nonreducing SDS gels. A significant portion of the peripherin/Rds-Rom-1 heterotetramers are linked together via intermolecular disulfide bonds to form hetero-octamers. Only some of the subunits within these hetero-octamers are disulfide bonded since both monomer and disulfide-linked dimers are observed on nonreducing SDS gels. Intermolecular disulfide bonds can form between two peripherin/Rds subunits, two Rom-1 subunits, or a peripherin/Rds and Rom-1 subunit. The hetero-octamers do not form higher order oligomers. Rom-1 homotetramers present in relatively low amounts do not readily form disulfide-linked oligomers.
amounts of rhodopsin by Western blotting and/or the use of less sensitive antibodies in these studies.

Peripherin/Rds and Rom-1 expressed in COS-1 cells show a similar pattern of disulfide-mediated oligomerization as found in ROS. A major fraction of peripherin/Rds core complex interacts through intermolecular disulfide bonds to form intermediate and higher order oligomers of various sizes. As in the case of ROS, all the subunits in these oligomers participate in intermolecular disulfide bond formation. Rom-1 expressed in COS-1 cells also self-assembles into a core complex, but this protein shows little tendency to form disulfide-linked oligomers, as observed in ROS. Mutagenesis studies have confirmed that disulfide-linked oligomerization of peripherin/Rds is mediated by Cys-150 present within the large intradiscal loop of the protein. The corresponding Cys-153 residue in Rom-1 is also likely to participate in intermolecular disulfide bond formation between heteromeric core complexes. However, in the absence of peripherin/Rds, two Cys-153 residues of Rom-1 show little tendency to form intermolecular disulfide bonds, possibly due to limited accessibility or unfavorable alignment of these groups.

These studies taken together indicate that peripherin/Rds exhibits a strong tendency to form disulfide-linked oligomers of various sizes. The association of peripherin/Rds with Rom-1 in the core complex limits the size of the oligomers and the number of subunits that participate in intermolecular disulfide bond formation (see Fig. 6). Thus, Rom-1 can be considered as a negative modulator of peripherin/Rds oligomerization.

Intermolecular disulfide bonds are known to be important in the assembly of multisubunit proteins and higher order oligomeric complexes. The light and heavy chains of immunoglobulins and the α and β subunits of the insulin receptor are joined by disulfide bonds. Intermolecular disulfide bonding is important in capsid assembly and disassembly of papillomavirus (36), stabilization of vaccinia virus (37), and oligomerization of tenascin-C, an extracellular matrix protein involved in embryogenesis and tumorigenesis (38).

We speculate that the disulfide-mediated oligomerization of peripherin/Rds plays an important role in outer segment disc morphogenesis and stabilization. This is based on the following. The cysteine residues responsible for intermolecular disulfide bonds (Cys-150 in peripherin/Rds and Cys-153 in Rom-1) are conserved in all vertebrate peripherin/Rds and Rom-1 proteins analyzed to date (29). Disulfide-linked dimerization of peripherin/Rds is a general property of these proteins (5, 7, 13, 26, 32). Sulfhydryl agents have been reported to disrupt new disc formation (39), and protein-disulfide isomerase, a protein that functions in the making and breaking of disulfide bonds, is present in ROS discs. In one model, peripherin/Rds and Rom-1 containing homomeric and heteromeric core complexes are envisioned to assemble in the endoplasmic reticulum membrane of photoreceptors and translocate in vesicles to the base of outer segments. Specific chaperone proteins may prevent disulfide-linked oligomerization from occurring during this trafficking process. At the base of the outer segment, protein-disulfide isomerase would catalyze disulfide-mediated oligomerization of peripherin/Rds containing homomeric and heteromeric core complexes across juxtaposed newly forming disc membranes to effectively zipper together the rim region as part of outer segment morphogenesis. The idea that membrane proteins can mediate membrane adhesion is not new. The major structural protein, Po, of peripheral nerve myelin is a tetrameric complex. The extracellular domains of these complexes on opposing membranes interact with each other to

mediate myelin membrane adhesion (40). In this case, however, protein-protein associations occur through noncovalent interactions.

An alternative mechanism would involve disulfide-linked oligomerization of peripherin/Rds containing core complexes laterally within a membrane. These oligomers could be envisioned to initiate the disc rim curvature or promote interactions with other outer segment proteins that participate in outer segment formation. Efforts are now underway to examine more directly the role of disulfide-mediated oligomerization of peripherin/Rds in disc morphogenesis.

Peripherin/Rds and Rom-1 differ significantly with respect to their role in outer segment morphogenesis. Peripherin/Rds is essential for outer segment morphogenesis since homozygous rds mice lacking this protein fail to form outer segments (10). Rom-1, on the other hand, appears to regulate the fine structure of the outer segment discs since homozygous rom-1 knockout mice produce outer segments with slightly enlarged discs (22). Disulfide-mediated oligomerization supports the dominant role of peripherin/Rds in outer segment disc morphogenesis. In addition to being more abundant than Rom-1 in ROS, peripherin/Rds is required for the formation of intermediate and higher order disulfide-linked oligomers, a process that may be crucial for disc morphogenesis as discussed above. Rom-1, on the other hand, does not form higher order oligomers, and therefore would not be considered to be essential for disc morphogenesis. Rom-1 may simply serve to limit the formation of higher order peripherin/Rds oligomer formation through interactions with peripherin/Rds and thereby regulate the size of the discs during outer segment morphogenesis.

A relatively large number of missense mutations in the large intradiscal loop of peripherin/Rds have been linked to a variety of human retinal degenerative diseases. Previous studies have indicated that some of these mutations affect protein folding and subunit assembly (28–29). A consequence of the misfolding of this large loop may be the inability of the peripherin/Rds mutants to form intermolecular disulfide bonds required for oligomerization. Such mutations in Rom-1 would have less impact since disulfide-linked oligomerization of Rom-1 is not crucial to outer segment morphogenesis and structure. In summary, we have shown here that peripherin/Rds and Rom-1 associate noncovalently to form homomeric and heteromeric core complexes. Peripherin/Rds containing complexes interact via Cys-150-mediated intermolecular disulfide bonds to form oligomers that may play an important role in rod and cone outer segment morphogenesis.

Acknowledgments—We thank Dr. Orson Moritz for providing the Rom1C6-Sepharose and Dr. Andrew Goldberg for helpful discussions during the early phase of this study.

REFERENCES

1. Steinberg, R. H., Fisher, S. K., and Anderson, D. H. (1980) J. Comp. Neurol. 190, 501–508
2. Roof, D. J., and Heuser, J. E. (1982) J. Cell Biol. 95, 487–500
3. Usukura, J., and Yamada, E. (1981) Biomed. Res. 2, 177–193
4. Curless, J. M., and Petter, R. D. (1987) J. Comp. Neurol. 257, 24–38
5. Molday, R. S., Hicks, D., and Molday, L. L. (1987) Invest. Ophthalmol. Vis. Sci. 28, 50–61
6. Connell, G., and Molday, R. S. (1990) Biochemistry 29, 4691–4698
7. Bascom, R. A., Manara, S., Collins, L., Molday, R. S., and Kalnins, V. I. (1992) Neuroeye 8, 1171–1184
8. Moritz, O. L., and Molday, R. S. (1996) Invest. Ophthalmol. Vis. Sci. 37, 152–262
9. Arikawa, K., Molday, L. L., Molday, R. S., and Williams, D. S. (1992) J. Cell Biol. 116, 659–667
10. Sanyal, S., and Jansen, H. G. (1981) Neurosci. Lett. 21, 23–26
11. Hawkins, R. R., Jansen, H. G., and Sanyal, S. (1985) Exp. Eye Res. 41, 701–720
12. Travis, G. H., Brennan, M. B., Danielson, P. E., Kazak, C. A., and Sutcliffe, J. G. (1989) Nature 336, 70–73
13. Connell, G., Bascom, R. A., Molday, L. L., Reid, D., McInnes, R. E., and Molday, R. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 723–726
14. Travis, G. L., Groshan, K. R., Lloyd, M., and Bok, D. (1992) Neuron 9, 115–119
15. Farrar, G. J., Reina, P., Jardan, S. A., Kumar-Singh, R., Humphries, M. M.,
16. Kaijawra, K., Hahn, L. B., Mukai, S., Travis, G. H., Berson, E. L., and Dryja, T. P. (1991) *Nature* **354**, 478–483

17. Wells, J., Wroblewski, J., Keen, J., Inglehearn, C., Jubb, C., Eckstein, A., Jay, M., Arden, G., Bhattacharya, S., Fitzke, F., and Bird, A. C. (1993) *Nat. Genet.* **3**, 213–218

18. Nicol, B. E., Sheffield, V. C., Vandenberg, K., Drack, A. V., Kimura, A. E., and Stone, E. M. (1993) *Nat. Genet.* **3**, 202–207

19. Wells, J., Wroblewski, J., Keen, J., Inglehearn, C., Jubb, C., Eckstein, A., Jay, M., Arden, G., Bhattacharya, S., Fitzke, F., and Bird, A. C. (1993) *Nat. Genet.* **3**, 213–218

20. Kajiwara, K., Berson, E. L., and Dryja, T. P. (1994) *Science* **264**, 1604–1608

21. Dryja, T. P., Carr, R. R., Murphy, W. H., Sheffield, V. C., and Stone, E. M. (1993) *Arch. Ophthalmol.* **111**, 1531–1542

22. Boesze-Battaglia, K., Lamba, O. P., Napoli, A. A., Sinha, S., and Guo, Y. (1998) *Biochemistry* **37**, 9477–9487

23. Dryja, T. P., Hahn, L. B., Kajiwara, K., and Berson, E. L. (1997) *Invest. Ophthalmol. Vis. Sci.* **38**, 498–509

24. Clarke, G., Goldberg, A. F. X., Vidgen, D., Collins, L., Ploder, L., Schwarz, L., Molday, R. S., Rossant, J., Szél, A., Molday, L. L., Birch, D. G., and McInnes, R. R. (2000) *Nat. Genet.*, in press

25. Molday, R. S. (1994) *Prog. Ret. & Eye Res.* **13**, 271–299

26. Travis, G. L., Sutcliffe, J. G., and Bok, D. (1991) *Neuron* **6**, 61–70

27. Boesze-Battaglia, K., Lamba, O. P., Napoli, A. A., Sinha, S., and Guo, Y. (1998) *Biochemistry* **37**, 9477–9487

28. Goldberg, A. F. X., and Molday, R. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13738–13740

29. Goldberg, A. F. X., Loewen, C. J. R., and Molday, R. S. (1998) *Biochemistry* **37**, 660–665

30. Goldberg, A. F. X., Moritz, O. L., and Molday, R. S. (1995) *Biochemistry* **34**, 14215–14219

31. Goldberg, A. F. X., and Molday, R. S. (1996) *Biochemistry* **35**, 6144–6149

32. Redzierski, W., Moghrabi, W. N., Allen, A. C., Jablonski-Stiemke, M. M., Azarian, S. M., Bok, D., and Travis, G. H. (1996) *J. Cell Biol.* **133**, 2551–2560

33. Molday, R. S., and Molday, L. L. (1987) *J. Cell Biol.* **105**, 2589–2601

34. Kaplan, R. S., and Pedersen, P. L. (1985) *Anal. Biochem.* **150**, 97–104

35. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379

36. Beard, L. M., Estes, P. A., Lyon, M. K., and Garcea, R. L. (1998) *J. Virol.* **72**, 2160–2167

37. Locker, J. K., and Griffiths, G. (1999) *J. Biol. Chem.* **274**, 2073–2077

38. Wetzel, M. G., Parsons, S. E., and Besharse, J. C. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**, 1519

39. Shapiro, L., Doyle, J. P., Hensley, P., Colman, D. R., and Hendrickson, W. A. (1996) *Neuron* **17**, 435–449
Disulfide-mediated Oligomerization of Peripherin/Rds and Rom-1 in Photoreceptor Disk Membranes: IMPLICATIONS FOR PHOTORECEPTOR OUTER SEGMENT MORPHOGENESIS AND DEGENERATION

Christopher J. R. Loewen and Robert S. Molday

J. Biol. Chem. 2000, 275:5370-5378.
doi: 10.1074/jbc.275.8.5370

Access the most updated version of this article at http://www.jbc.org/content/275/8/5370

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 15 of which can be accessed free at http://www.jbc.org/content/275/8/5370.full.html#ref-list-1