Heterologous Expression of Photoreceptor Peripherin/*rds* and Rom-1 in COS-1 Cells: Assembly, Interactions, and Localization of Multisubunit Complexes†

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ABSTRACT: Peripherin/*rds* is a 39 kDa integral membrane glycoprotein essential for normal photoreceptor cell development in vertebrates. It has been implicated in several human retinal degenerative diseases including retinitis pigmentosa and macular degeneration and is thought to play a structural role at photoreceptor outer segment disk rims, where it forms a tightly-associated complex with rom-1, a nonglycosylated 37 kDa homologue. Western blot analysis of COS-1 cells transiently transfected with full-length cDNA coding for either peripherin/*rds* or rom-1 indicates that each protein is expressed primarily as a disulfide-linked homodimer; recombinant peripherin/*rds* is glycosylated while recombinant rom-1 is not—akin to their counterparts in rod photoreceptor disk membranes. Upon cotransfection of the two cDNAs, the specific assembly of a stable peripherin/*rds*−rom-1 complex is observed. Immunofluorescence microscopy studies demonstrate that both singly and coexpressed peripherin/*rds* and rom-1 complexes are localized primarily within internal membranes of transfected cells. Velocity sedimentation data indicate that the recombinant complexes (4.9 S) are assembled with a subunit stoichiometry similar to those extracted from ROS membranes (4.5 S) and are most consistent with a tetrameric arrangement of polypeptides. Sedimentation analyses of individually expressed peripherin/*rds* (5.1 S) and rom-1 (4.3 S) suggest that each polypeptide can also assemble into a tetrameric form in the absence of its homologue partner. Subunit assembly and interactions are discussed in terms of their potential role in hereditary retinal diseases.

Peripherin/*rds* is an integral membrane glycoprotein found in the outer segment disks of vertebrate rod and cone photoreceptors (Connell & Molday, 1990; Travis et al., 1991; Arikawa et al., 1992; Molday, 1994). It is believed to serve as an essential structural element in the formation and maintenance of normal outer segments; however, its functional activity at the molecular level is not presently defined. Interest in this protein has mounted considerably as instances of retinal degeneration in humans have been linked to mutations in the *rds* gene [Farrar et al., 1991; Kajiwara et al., 1991, 1993, 1994; Meins et al., 1993; Nichols et al., 1993a,b; Saga et al., 1993; Wells et al., 1993; Fishman et al., 1994; Gruning et al., 1994; Wroblewski et al., 1994; Gorin et al., 1995; for reviews, see: Davies (1993) and Molday (1994)]. The mechanism(s) by which mutations in the gene for peripherin/*rds* result in photoreceptor cell degeneration is (are) not known, and one current line of research is the explicit identification of the molecular pathways which culminate in photoreceptor degeneration.

The immunocytochemical localization of peripherin/*rds* initially led to the suggestion that it functions as a structural element in outer segment disk rims (Molday et al., 1987). Evidence in support of this hypothesis is provided by the homozygous *rds* mouse model, which contains an insertion of foreign DNA within an exon of the *rds* gene and develops photoreceptor cells which fail to form outer segments (Van Nie et al., 1978; Sanyal & Jansen, 1981; Travis et al., 1989). Transgenic rescue of *rds* mice with a normal copy of the *rds* gene results in the restoration of outer segments and the prevention of photoreceptor degeneration (Travis et al., 1992).

Peripherin/*rds* has been implicated in a noncovalent protein−protein interaction with rom-1, a nonglycosylated homologue which appears to share a similar outer segment localization, membrane topology, and overall structure (Bascom et al., 1992, 1993). Rom-1 has been reported in human rod, but not cone, photoreceptor cells, and has been suggested to play a role in the differing morphologies of rods and cones (Bascom et al., 1992). Although these proteins are believed to play crucial roles in normal outer segment structure and human retinal disease, the functional activities of peripherin/*rds* and rom-1 at the molecular level are not currently understood.

Heterologous expression systems have proven valuable for the molecular-level characterization of both wild-type and disease-linked proteins; in particular, mammalian cell lines have been used widely for the expression of integral membrane proteins (Lefkowitz & Caron, 1988; Lemas et al., 1992; Gu et al., 1990), including photoreceptor proteins (Oprian et al., 1987; Sung et al., 1991; Chen et al., 1993). In this report, we examine the expression, localization, subunit assembly and interactions of the wild-type peripherin/*rds* and rom-1 proteins expressed in COS-1 cells as an important step toward elucidating their roles in both normal outer segment morphogenesis and degenerative retinal diseases.

METHODS

Construction of Expression Plasmids. An *Eco*RI fragment containing the full-length coding region of bovine peripherin/

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rds (Connell et al., 1991) was subcloned into the mammalian expression vector pcDNAI/AMP (Invitrogen Corp., San Diego, CA) and designated pcPER. Proper orientation of the peripherin/*rds* gene was selected by restriction mapping. A synthetic gene coding for bovine rhodopsin (Oprian et al., 1987) was subcloned as an *Eco*RI/*Not*I fragment into the polylinker region of pcDNAI/AMP (pcRHO). The construction of a pcDNAI/AMP plasmid containing the rom-1 coding sequence (pcROM) has been described (Moritz et al., 1994).

Cell Culture and Transfection. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/mL penicillin G, and 50 μg/mL streptomycin at 37 °C, 5% CO_2. Transfections by electroporation, calcium phosphate, or DEAE-dextran all gave satisfactory peripherin/*rds* expression (>0.5% of total Triton X-100 extractable protein) by Western blot analysis. Since calcium phosphate transfection produced substantially higher rom-1 expression levels, and yielded a greater percentage of peripherin/*rds*−rom-1 coexpressing cells (by immunofluorescent labeling), it was chosen for coexpression studies. Although expression levels for individually expressed proteins gave a simple dependence upon the concentration of the transfected plasmid, levels for coexpressed proteins did not and were interdependent in a complex manner. Cotransfections were performed with equimolar amounts of each plasmid unless otherwise indicated. Tri-transfections utilized a mixture of pcPER, pcROM, and pcRHO (2:6:1 mass ratio) to minimize differences in protein expression levels.

Calcium phosphate transfections were performed in 60 mm dishes essentially as described (Chen & Okayama, 1987) using 9−10 μg of total plasmid DNA per 2 × 10^5 cells. Transfectants harvested 48 h posttransfection for immunofluorescence, Western, or autoradiographic analyses were prepared as described below.

Western Blot Analysis and Enzymatic Deglycosylation. COS-1 cells were washed twice with phosphate-buffered saline (PBS)[1] while still attached to tissue culture dishes. Cellular extracts were prepared by application of 300 μL of extraction buffer (1× PBS, 1.0% Triton X-100, 1 mM DTT, and 100 μg/mL PMSF, pH 7.4) to each 60 mm diameter dish, for 20 min at 4 °C. Extracts were centrifuged for 20 min at 14000*g* or 100000*g* and the postnuclear supernatant fractions collected and maintained at 4 °C prior to use.

Polyacrylamide gels (10%) were used essentially as described (Laemmli, 1970); however, to avoid membrane protein aggregation, samples were not heated prior to loading. Proteins transferred to Immobilon-P membranes (Millipore, Bedford, MA) by tank or semi-dry electroblotting were probed with primary antibodies from hybridoma culture supernatants using standard methods (Harlow & Lane, 1988). Monoclonal antibodies per2B6 (Molday et al., 1987), rho1D4 (Hodges et al., 1988), and rom1D5 and polyclonal antibody romC2 (Moritz et al., 1994) have been described previously. Primary antibody binding was identified with secondary antibodies conjugated to horseradish peroxidase followed by chemiluminescent detection (ECL; Amersham Canada Ltd., Oakville, Ontario).

[1] Abbreviations: β-ME, β-mercaptoethanol; BSA, bovine serum albumin; DTT, dithiothreitol; Endo H, endoglycosidase H; FITC, fluorescein isothiocyanate; Fmoc, fluorenylmethoxycarbonyl; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; ROS, rod outer segments; TRITC, tetramethylrhodamine isothiocyanate.

Samples to be deglycosylated were treated with 200 units of recombinant endoglycosidase H_f (New England Biolabs, Beverly, MA) per microgram of protein, for 2 h at 37 °C prior to SDS−PAGE and Western blotting.

Immunofluorescence Microscopy. Cells destined for microscopic analysis were plated on glass coverslips prior to transfection and fixed for labeling approximately 48 h posttransfection by immersion in methanol for 1 h at 4 °C. Coverslips were washed once with PBS, blocked with 4% BSA, 1× PBS for 30 min, and then labeled with primary antibodies (diluted in 0.4% BSA, 1× PBS) for 1 h at room temperature. Rom1D5 (diluted 1:150) and rho1D4 (diluted 1:80) were used as hybridoma culture supernatants, and were detected by an FITC-labeled anti-mouse secondary antibody (diluted 1:50; Cedarlane Laboratories, Hornby, Ontario) to localize expressed rom-1 and rhodopsin, respectively. Monoclonal antibody per2B6 was purified from ascities fluid (described below), fluorescently labeled with TRITC (Harlow & Lane, 1988), and used for peripherin/*rds* localization at 5 μg/mL. Double-labeling of coexpressing cells was performed by (1) indirect labeling with rom1D5 or rho1D4 and FITC-secondary antibody, (2) quenching unreacted secondary antibody sites with mouse sera (10× diluted in 0.4% BSA, 1× PBS) for 1 h at room temperature, and (3) direct labeling with TRITC-per2B6. Control studies indicated that antibody labeling was dependent upon transfection with the appropriate expression plasmid and could be entirely and specifically inhibited by the inclusion of synthetic peptides corresponding to the primary antibody epitopes. Peptides (nine amino acids each) corresponding to the per2B6 (Connell et al., 1991) and rho1D4 (Hodges et al., 1988) epitopes have been described. An 11 amino acid peptide corresponding to the rom1D5 epitope, LFPAGLKDMLK (Moritz et al., 1994), was synthesized by standard Fmoc solid phase chemistry (LKB Biolynx 4175 peptide synthesizer) and used without further purification.

Immunoprecipitations and Metabolic Labeling. Monoclonal antibodies per2B6, rho1D4, and rom1D5 were purified from ascites fluids by ammonium sulfate precipitation and ion-exchange chromatography and covalently coupled to CNBr-activated Sepharose-2B at approximately 2 mg of protein/mL of beads as described (Connell et al., 1991). In general, a 10-fold volume excess of COS-1 cell detergent lysate (300 μL) was combined with the indicated immobilized antibody (ca. 30 μL of beads) and gently mixed at 4 °C for 1 h or overnight. After removal of supernatants, beads were washed 3 times with a 30-fold volume excess of wash buffer (1× PBS, 0.1% Triton X-100, and 1 mM DTT, pH 7.4) and then eluted with an equal volume of 2.5× Laemmli loading buffer for 10 min at room temperature.

Cells were metabolically labeled at 37 °C in 5% CO_2 for 6 h in cysteine- and methionine-free DMEM (+5% dialyzed FBS) containing ca. 200 μCi/mL [^{35}S]methionine and [^{35}S]-cysteine (Trans^{35}S-label; ICN Biomedicals Canada Ltd., Mississauga, Ontario) beginning approximately 48 h posttransfection. Immunoprecipitations performed upon detergent extracts prepared from these cells were resolved by SDS−PAGE and detected by autoradiography.

Immunopurification for Sedimentation Analysis. Peripherin/*rds*, rom-1, and peripherin/*rds*−rom-1 complexes were isolated from two (singly transfected) or six (cotransfected) 60 mm dishes of COS-1 cells with either per2B6−Sepharose or rom1D5−Sepharose essentially as described above.

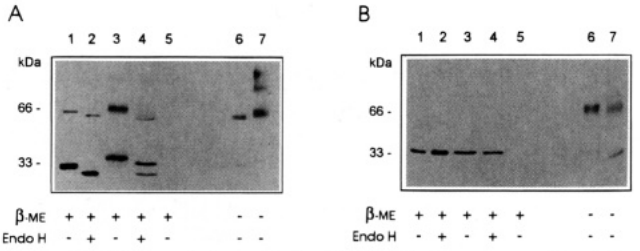

FIGURE 1: Western blot analysis of individually expressed peripherin/*rds* and rom-1 proteins in COS-1 cells. Detergent extracts of COS-1 cells transiently transfected with either pcPER (panel A, lanes 3, 4, and 7), pcROM (panel B, lanes 3, 4, and 7), or vector alone (each panel, lane 5) were electrophoresed under reducing or nonreducing (±β-ME) denaturing conditions, and then Western blotted with monoclonal antibodies specific for peripherin/*rds* (per2B6, panel A) or rom-1 (rom1C2, panel B). Mobility shifts induced by Endo H indicate high-mannose or hybrid carbohydrate modifications. A detergent extract from ROS membranes is shown for comparison in lanes 1, 2, and 6 (each panel).

Proteins were specifically eluted from the immunoaffinity matrix under nondenaturing conditions for 1 h at room temperature by the addition of a 2-fold bead volume of elution buffer (1× PBS, 0.1% Triton X-100, 1 mM DTT, and 0.1 mg/mL peptide), containing a peptide (described above) corresponding to the immobilized antibody epitope.

Sedimentation Velocity Analysis. Sedimentation analysis was performed in 5−20% (w/w) sucrose gradients containing 1× PBS, 0.1% Trition X-100, and 1 mM DTT, pH 7.4, essentially according to the method of Martin and Ames (1961), assuming a partial specific volume of 0.8 cm^3/g. Sedimentation coefficients were converted to $S_{20,w}$ values using density and estimated viscosity at half the radial distance traveled. Control experiments on lysozyme and bovine serum albumin yielded sedimentation velocities within 10% of literature values.

Samples (75−150 μL) were loaded onto 2 mL linear gradients and centrifuged at 50K rpm, 4 °C, for 16 h in a Beckman TLS-55 rotor, and fractions of approximately 100 μL were collected by piercing the bottoms of the centrifuge tubes. Sucrose concentration was assayed by refractometry and individual proteins by laser densitometry (LKB Ultroscan XL) of Coomassie-blue stained SDS−polyacrylamide gels or Western blots. Peripherin/*rds*−rom-1 complexes were specifically identified by a two-step protocol: (1) immunopurification and sedimentation of complexes containing peripherin/*rds* (per2B6), followed by (2) Western blot analysis of fractions for rom-1 reactivity (rom1D5). Identical results were obtained when complexes were rom1D5-immunopurified and sedimentation fractions probed for per2B6 reactivity.

RESULTS

Heterologous Expression of Peripherin/rds and Rom-1. Postnuclear lysates from transiently transfected COS-1 cells were compared to a detergent extract of ROS membranes by Western blot analysis using anti-peripherin/*rds* monoclonal antibody per2B6 (Figure 1A). Only cells transfected with pcPER show peripherin/*rds* reactivity; lysates from mock-transfected cells do not. The expressed protein (lane 3) migrates slightly more slowly by SDS−PAGE relative to that from ROS membranes (lane 1), likely due to altered glycosylation; in each instance, treatment with Endo H (an endoglycosidase specific for high-mannose or hybrid-type N-linked carbohydrates) produces a core polypeptide of ca. 31 kDa (lanes 2 and 4). Residual amounts of Endo H resistant protein are variable between transfections and may represent altered or incompletely processed products. In the absence of an added reducing agent, the heterologously expressed peripherin/*rds* runs primarily in a dimerized form at ca. 66 kDa, akin to that found in ROS extracts (lanes 6 and 7).

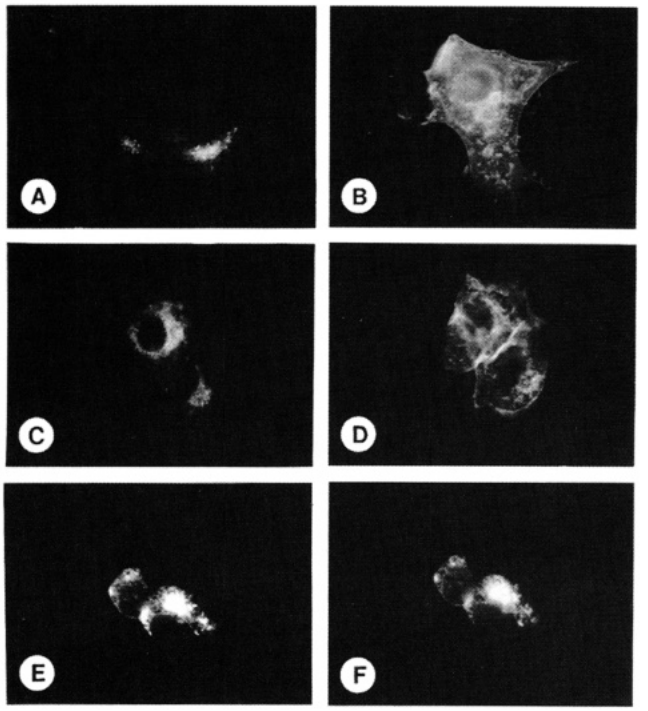

FIGURE 2: Immunofluorescent localization of expressed photoreceptor proteins: peripherin/*rds*, rom-1, and rhodopsin. COS-1 cells individually transfected with either pcPER or pcRHO and labeled with anti-peripherin/*rds* or anti-rhodopsin antibodies illustrate contrasting localization patterns for peripherin/*rds* (A) and rhodopsin (B). Double-labeling of a single pair of cells cotransfected with both pcPER and pcRHO indicates that coexpression does not affect the localization of either peripherin/*rds* (C) or rhodopsin (D). Double-labeling of cells cotransfected with pcPER and pcROM demonstrates that rom-1 is localized to internal cell membranes (F) and does not affect peripherin/*rds* localization (E). All immunofluorescent labelings were dependent upon transfection with the appropriate plasmid(s) and could be specifically inhibited by the addition of peptides corresponding to primary antibody epitopes (not shown).

A similar analysis was performed for the homologous protein rom-1 (Figure 1B). Rom-1 antibody reactivity is not observed for mock-transfected cell lysates, but a band of mobility similar to rom-1 from ROS membranes is identified in pcROM-transfected lysates (lanes 1 and 3). In contrast to peripherin/*rds*, but like rom-1 from ROS, no N-linked carbohydrate modification is apparent—endoglycosidase treatment does not affect its SDS−PAGE mobility (lanes 2 and 4). In the absence of reducing agent, recombinant rom-1 also demonstrates the ability to form disulfide-linked homodimers, akin to the authentic protein (lanes 6 and 7).

Immunofluorescent Localization of Expressed Peripherin/rds and Rom-1. Fluorescently labeled monoclonal antibody per2B6 was utilized to examine the subcellular localization of expressed peripherin/*rds* in permeabilized COS-1 cells (Figure 2). Individually expressed peripherin/*rds* is observed primarily within internal membranes, in contrast to rhodopsin expressed in COS-1 cells, which appears to label primarily

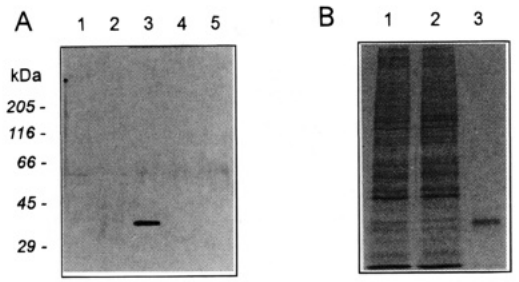

FIGURE 3: Immunoprecipitation of peripherin/*rds*−rom-1 complexes assembled by cotransfected COS-1 cells. Detergent lysates of COS-1 transiently transfected with the indicated plasmids were immunoprecipitated with anti-peripherin/*rds* antibody per2B6, and immunoprecipitation reactions were tested for coprecipitating rom-1 reactivity by Western blot analysis with anti-rom-1 antibody rom1C2 (panel A). Lane 1, pcPER; lane 2, pcROM; lane 3, cotransfected pcPER and pcROM; lane 4, cotransfected pcPER and pcROM (immunoprecipitation was inhibited with a peptide corresponding to the per2B6 epitope); lane 5, individually transfected pcPER and pcROM (combined detergent lysates seen in lanes 1 and 2). Panel B shows an autoradiogram of an anti-peripherin/*rds* (per2B6) immunoprecipitation reaction from COS-1 cells cotransfected with pcPER/pcROM and metabolically labeled with [^{35}S]-methionine and [^{35}S]cysteine (lane 3). Lane 1, total detergent lysate; lane 2 unbound fraction. The band in lane 3 contains comigrating peripherin/*rds* and rom-1 proteins.

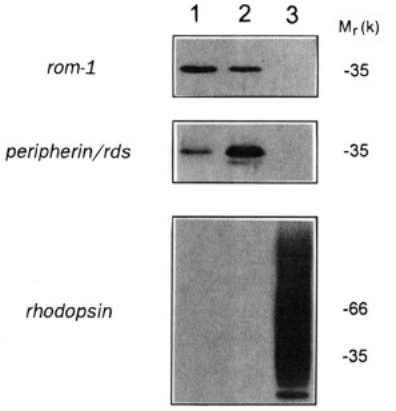

FIGURE 4: Specificity of peripherin/*rds*−rom-1 interaction in COS-1 cells. Rom-1 (lane 1), peripherin/*rds* (lane 2), and rhodopsin (lane 3) were individually immunoprecipitated (see Methods) from a detergent lysate of COS-1 cells transfected with a mixture of the three expression plasmids. The immunoprecipitation reactions were Western blotted and probed with antibodies against rom-1 (romC2), peripherin/*rds* (per2B6), and rhodopsin (rho1D4) as indicated. Although rom-1 and peripherin/*rds* are observed to coprecipitate each other (an excess of uncomplexed peripherin/*rds* appears to be present), interactions with rhodopsin are not observed. Heterogeneous glycosylation has been proposed to account for the broad smear of reactivity characteristic of expressed and purified rhodopsin (Doi et al., 1990).

at cell surfaces with anti-rhodopsin monoclonal antibody rho1D4 (Figure 2, panels A and B). Labeling of each protein is dependent upon transfection with its corresponding cDNA and is completely and specifically inhibitable by inclusion of peptides corresponding to the antibody epitopes. Similar results are observed for singly expressing stable transfectants of Chinese hamster ovary cells (data not shown).

Since peripherin/*rds* and rom-1 are known to exist as a multipolypeptide complex in ROS membranes, we tested whether rom-1 affects the translocation and localization of peripherin/*rds* in the COS-1 cell system. Cells coexpressing either peripherin/*rds* and rom-1 or peripherin/*rds* and rhodopsin were immunofluorescently double-labeled for the expressing proteins. Coexpression of peripherin/*rds* with rom-1 results in their colocalization to internal membranes (Figure 2, panels E and F); peripherin/*rds* localization is not affected by the presence of its homologue. Coexpression of peripherin/*rds* with rhodopsin results in localization of peripherin/*rds* to internal membranes, while rhodopsin is translocated to the plasma membrane (Figure 2, panels C and D).

Immunoprecipitation of Peripherin/rds−Rom-1 Complexes. Previous studies have documented a strong association between peripherin/*rds* and rom-1 in nonionic detergents (Bascom et al., 1992). To examine whether this interaction is a general feature of these proteins (as opposed to a unique aspect of the photoreceptor environment), peripherin/*rds* and rom-1 were coexpressed in the COS-1 system and assayed for complex formation by probing Western blots of anti-peripherin/*rds* immunoprecipitation reactions with an anti-rom-1 antibody. If a strong interaction is maintained by the heterologously expressed proteins, then immunoprecipitation of peripherin/*rds* is predicted to result in the specific coprecipitation of rom-1.

No precipitation of rom-1 is observed when per2B6 immunoprecipitations are performed on detergent extracts from cells expressing either peripherin/*rds* or rom-1 alone (Figure 3A, lanes 1 and 2); however, per2B6 immunoprecipitation of extracts from cotransfected cells results in a coprecipitation of rom-1 (Figure 3A, lane 3). Examination of the unbound fraction indicates that nearly all of the rom-1 expressed in calcium phosphate cotransfected cells is associated with peripherin/*rds* (not shown). Coprecipitation of rom-1 is dependent upon its association with peripherin/*rds*; it is completely inhibited by inclusion of a nine amino acid peptide corresponding to the anti-peripherin/*rds* 2B6 epitope (Figure 3A, lane 4). Furthermore, association between the expressed proteins appears to require coexpression, since mixing of cellular lysates containing individually expressed proteins is not sufficient for complex formation (Figure 3A, lane 5). These results demonstrate the assembly of a stable peripherin/*rds*−rom-1 complex under cotransfection conditions.

To characterize the specificity of assembly, i.e., whether the peripherin/*rds*−rom-1 complex is associated with endogenous COS-1 proteins, per2B6 immunoprecipitations were performed from detergent lysates of cotransfected cells metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Figure 3B (lane 3) shows a single immunoprecipitating major band at a molecular mass of ca. 33 kDa, corresponding to the mobility of the heterologously expressed peripherin/*rds* and rom-1 proteins. This result demonstrates that the assembly of peripherin/*rds*−rom-1 complexes in cotransfected COS-1 cells is specific; stable associations with endogenous COS-1 cell proteins are not observed.

The specificity of heterologous protein assembly in the COS-1 system was further examined by determining whether peripherin/*rds* associates with rhodopsin, a protein present in photoreceptor outer segment disk membranes in great abundance, although not associated with peripherin/*rds*. Detergent extracts from cells cotransfected with peripherin/*rds*, rom-1, and rhodopsin were analyzed by immunoprecipitation and Western blotting for potential protein−protein interactions (Figure 4). Immunoprecipitation of peripherin/*rds* results in the coprecipitation of a large fraction of the expressed rom-1 (upper panel, lane 2 vs 1). The converse

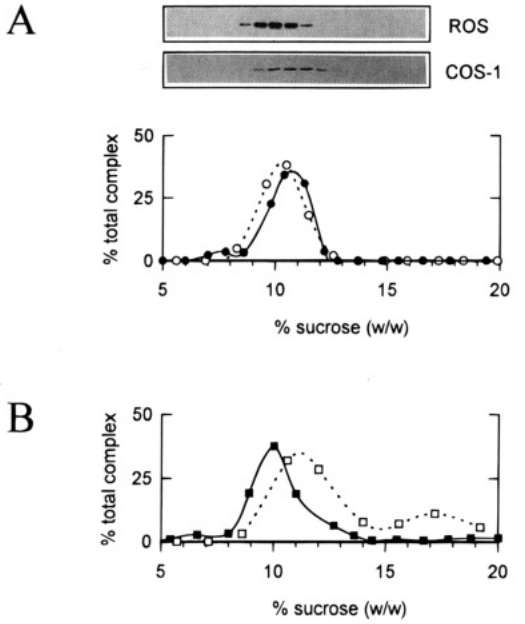

FIGURE 5: Sedimentation velocity analysis of complexes formed by peripherin/*rds* and rom-1 in bovine ROS membranes and COS-1 cells. Peripherin/*rds*−rom-1 complexes from Triton X-100 solubilized bovine ROS membranes (○) and per2B6-immunopurified complexes from cotransfected COS-1 cells (●) show similar sedimentation velocities in 5−20% (w/w) sucrose gradients (panel A). Chemiluminescent Western blots using anti-peripherin antibody per2B6 (ROS) and anti-rom-1 antibody romC2 (COS-1) are shown, as are corresponding plots of laser densitometric scans. Plots of individually COS-1 expressed peripherin/*rds* (□) and rom-1 (■) proteins were derived in an analogous manner and are shown in panel B. Sedimentation values were estimated as described under Methods and are reported under Results.

is also observed—immunoprecipitation of rom-1 results in the coprecipitation of peripherin/*rds* (middle panel, lane 1 vs 2). An excess of uncomplexed peripherin/*rds* is apparent, which varies between individual transfections and is likely a product of relatively low levels of rom-1 expression. In neither instance is rhodopsin observed as a coprecipitant (lower panel, lanes 1 and 2), although it is expressed at relatively high levels. Furthermore, immunoprecipitation of rhodopsin from these lysates does not result in a measurable coprecipitation of either peripherin/*rds* or rom-1 (upper and middle panels, lanes 3). These results strongly suggest that the assembly of peripherin/*rds* with rom-1 in COS-1 cells is a process which is specific with respect to exogenous as well as endogenous proteins.

Velocity Sedimentation of the Peripherin/rds−Rom-1 Complex. The fidelity of heterologous assembly was examined by velocity sedimentation analysis of recombinant complexes, in comparison to the peripherin/*rds*−rom-1 complex from detergent-solubilized ROS membranes. Figure 5A depicts a sedimentation profile of the ROS complex in a 5−20% sucrose density gradient, assayed for peripherin/*rds* by Western blotting. The Triton X-100 solubilized peripherin/*rds*−rom-1 complex from bovine ROS migrates with an apparent sedimentation coefficient of 4.5 ± 0.3 S ($n = 5$ ± SD). Purification of the complex by immunoaffinity chromatography has no effect upon its sedimentation mobility, suggesting the lack of additional strongly interacting ROS components in the solubilized state. Detection of complexes using an anti-rom-1 antibody yielded identical results (data not shown).

The sedimentation velocity of immunopurified peripherin/*rds*−rom-1 complexes from cotransfected COS-1 cell lysates was determined in an analogous manner (Figure 5A); assembled complexes were identified by sequential immunopurification (via per2B6) and detection (via romC2) with antibodies of differing reactivities. Recombinant complexes migrated with an apparent sedimentation coefficient of 4.9 ± 0.3 S ($n = 4$ ± SD), just slightly faster than the native ROS complex. Identical results were obtained when immunopurification and sedimentation of rom-1-containing complexes were analyzed by Western blots probed with an anti-peripherin/*rds* antibody. Analysis of the sedimented complexes from COS-1 cells by nonreducing SDS−PAGE confirms that peripherin/*rds* and rom-1 are present primarily as dimers (like those in ROS). These results indicate that recombinant peripherin/*rds*−rom-1 complexes are assembled with a stoichiometry akin to that of the authentic ROS complex.

Velocity Sedimentation of Individually Expressed Peripherin/rds and Rom-1. Since certain forms of human retinal degenerative diseases appear to result from the partial absence of either peripherin/*rds* or rom-1, we examined the sedimentation behavior of these proteins expressed individually. Figure 5B depicts a sedimentation velocity profile for Triton X-100 solubilized and immunopurified peripherin/*rds* expressed in COS-1 cells. Interestingly, peripherin/*rds* sediments at 5.1 S (mean, $n = 2$), similar to its value when in complex with rom-1. Individually expressed rom-1 also appears to be expressed in a similar stoichiometric form, sedimenting with an *s*-value of 4.3 S (mean, $n = 2$). These results indicate that even when expressed individually, both peripherin/*rds* and rom-1 form native-like multimeric complexes, in the absence of their homologue partners. This observation indicates that homodimers of either polypeptide can self-associate to form potentially functional complexes.

DISCUSSION

This paper reports the development of a heterologous expression system (COS-1) for the study of the peripherin/*rds*−rom-1 complex from vertebrate photoreceptors. We demonstrate the expression and localization of recombinant peripherin/*rds* and rom-1 proteins and their assembly into a multisubunit complex which by all available criteria resembles the complex from ROS.

Immunofluorescence data demonstrate an internal localization for both individually expressed peripherin/*rds* and rom-1 and assembled peripherin/*rds*−rom-1 complexes in COS-1 cells. Individual cells, shown to translocate rhodopsin to their plasma membrane, retain most, if not all, peripherin/*rds* and rom-1 within internal membranes. It is uncertain as to where localization should be expected, since organelles analogous to photoreceptor outer segment disks are not present in COS-1. In the vertebrate photoreceptor system, rhodopsin is present in both disk and plasma membranes, but peripherin/*rds* and rom-1 are localized solely to the rim regions of disk membranes (Molday et al., 1987; Arikawa et al., 1992; Bascom et al., 1992). Furthermore, Fariss et al. (1993) have shown that in detached retina rhodopsin redistributes along the plasma membrane of the entire photoreceptor cell, whereas peripherin/*rds* accumulates in internal perinuclear membranes. These observations suggest that peripherin/*rds* and rom-1 may contain targeting signals

which specifically prevent their localization to photoreceptor (and COS-1) plasma membranes.

Plasma membrane targeting is thought to be the default pathway for membrane proteins in eukaryotic cells (Rothman & Orci, 1992); hence, lack of export to the plasmalemma may indicate improper folding of the protein (Doi et al., 1990; Sung et al., 1991, 1993). Internal localization, however, is not synonymous with misfolding; in several instances, heterologously expressed proteins retained in internal membranes are found to be fully functional (Ruetz et al., 1993; Pasyk & Foskett, 1995). Our findings suggest that the internal localization of peripherin/*rds* and rom-1 in COS-1 cells does not reflect improper biosynthesis or misfolding, but rather may indicate the presence of targeting signals which prevent their translocation to the plasma membrane. This observation may provide a starting point for the analysis of sorting signals responsible for targeting of some photoreceptor proteins to the rim regions of outer segment disk membranes.

Immunoprecipitation experiments demonstrate that the study of heterologously expressed peripherin/*rds* and rom-1 is feasible in the COS-1 cell line. Lacking a *bona fide* functional assay, we have demonstrated that the recombinant proteins are structurally similar to authentic ROS proteins by several criteria, the most compelling of which is their assembly into a multisubunit complex of native size and composition. Assembly is specific; stable associations with endogenous COS-1 proteins, or another heterologously expressed photoreceptor membrane protein (rhodopsin), are not observed. Moreover, the interaction between peripherin/*rds* and rom-1 requires coexpression; reconstitution of cellular extracts which contain the singly expressed proteins (at concentrations severalfold higher relative to coexpressed proteins) does not result in measurable association. Although it is not possible to rigorously assign molecular weights to integral membrane (detergent binding) proteins from velocity sedimentation data alone, the major sedimenting peripherin/*rds*–rom-1 species we observe is most consistent with a tetrameric arrangement of polypeptides (152 kDa complex)—association of a disulfide-linked peripherin/*rds* homodimer with a disulfide-linked rom-1 homodimer. Initial data from a complete hydrodynamic characterization of the Triton X-100 solubilized ROS complex support this interpretation (Goldberg and Molday, unpublished experiments).

Recombinant complexes are assembled with a native stoichiometry when peripherin/*rds* and rom-1 proteins are coexpressed. It may be concluded that the information required for faithful complex formation is a general property of the two polypeptides and not a unique aspect of the photoreceptor environment. Interestingly, we find that in the absence of rom-1, peripherin/*rds* sediments at the same rate as the peripherin/*rds*–rom-1 complex, suggesting that two peripherin/*rds* homodimers can assemble to form a tetrameric complex. Likewise, in the absence of peripherin/*rds*, rom-1 appears as a tetrameric form. Reconstitution of the individually expressed proteins does not result in measurable interaction (on a time scale of hours); hence, once formed, the detergent-solubilized homotetrameric complexes are tightly-associated, and subunit exchange does not occur. Since homomeric forms of these proteins do not appear to be present in photoreceptor membranes (Bascom et al., 1992; Moritz et al., 1994), these findings imply a strict regulation of heteromeric assembly in normal photoreceptor cells. The observation that a peripherin/*rds* homodimer can recognize *either* a rom-1 homodimer or another peripherin/*rds* homodimer, but not both simultaneously, suggests a simple model in which the sites of interaction for both hetero- and homotypic association are identical or overlapping. These findings suggest that sites likely to mediate peripherin/*rds*–rom-1 interaction may be found within structural motifs conserved between the two polypeptides. Although high degrees of conservation are observed in small regions of the large intradiskal loops for each polypeptide (Bascom et al., 1992), it is also possible that intersubunit contacts rely upon hydrophobic interactions within transmembrane segments. Mutagenic analysis using the expression system developed here is in progress to determine intersubunit contact regions.

Several observations suggest that the peripherin/*rds*–rom-1 complex is best understood as an association of subunits within a single multimeric protein, rather than a protein–protein interaction between distinct proteins: the two molecules display homologous amino acid sequences, similar folding patterns and orientation in outer segment disk membranes, and a stoichiometric association in ROS membranes (Bascom et al., 1992; Moritz et al., 1994). The present findings, that heterologously expressed polypeptides require *in vivo* assembly for interaction, reinforce the notion that these molecules are subunits of a larger functional unit, rather than truly independent (and interacting) proteins in and of themselves. A similar scenario has emerged for the photoreceptor cGMP-dependent cation channel. Initially described as a homomultimer (Kaupp et al., 1989), more recent reports detail the existence of a tightly-associated second subunit, which can modify the function of heterologously expressed heteromeric channels, yet does not form functional channels itself (Chen et al., 1993).

Although normally found as heterotetramers in bovine photoreceptors, the present findings indicate that each subunit is also structurally competent to assemble into a homomeric form, i,e., (peripherin/*rds*)$_4$ or (rom-1)$_4$, in the absence of the other. This observation has important implications for human retinal degenerative diseases linked to putative null mutations in these proteins. In at least one instance, a null mutation in rom-1 does not appear to induce a disease phenotype (Kajiwara et al., 1994). It is tempting to speculate that (peripherin/*rds*)$_4$ is formed in these individuals and can compensate for normally heteromeric, (peripherin/*rds*)$_2$–(rom-1)$_2$ complexes. In contrast, null mutations in peripherin/*rds* have been linked to disease phenotypes in humans (Kajiwara et al., 1993; Meins et al., 1993), suggesting that if rom-1 homomeric complexes (rom-1)$_4$ are formed, they are not competent to offset a lack of functional heteromeric complexes. Biochemical-level analysis of transgenic mice which lack rom-1 or peripherin/*rds* can be utilized to directly test this model.

We have shown that heterologous expression of peripherin/*rds* and rom-1 in the COS-1 cell line is a valuable means for studying the wild-type proteins. Our results argue that these polypeptides do not normally act independently, but rather constitute subunits of a larger functional unit, which preliminary evidence suggests is tetrameric. Furthermore, we show that each subunit, in the absence of its homologue partner, assumes a native-like multimeric form, suggesting that homomultimers may function in abnormal (mutant) photoreceptor physiology. Finally, we expect this system to prove useful for the examination of particular mutations

in the *rds* and rom-1 genes, which have been linked to retinal degenerative diseases (including retinitis pigmentosa and macular degenerations), and may cause changes in the normal subunit structure and assembly of the peripherin/*rds*−rom-1 complex.

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