Retinitis Pigmentosa GTPase Regulator (RPGR)-interacting Protein Is Stably Associated with the Photoreceptor Ciliary Axoneme and Anchors RPGR to the Connecting Cilium*

Received for publication, October 13, 2000, and in revised form, November 16, 2000
Published, JBC Papers in Press, December 4, 2000, DOI 10.1074/jbc.M009351200

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Retinitis pigmentosa (RP) is a blinding retinal disease in which the photoreceptor cells degenerate. Mutations in the gene for retinitis pigmentosa GTPase regulator (RPGR) are a frequent cause of RP. The function of RPGR is not well understood, but it is thought to be a putative guanine nucleotide exchange factor for an unknown G protein. Ablation of the RPGR gene in mice suggested a role in maintaining the polarized distribution of opsins across the cilia. To investigate its function, we used a protein interaction screen to identify candidate proteins that may interact physiologically with RPGR. One such protein, designated RPGR-interacting protein (RPGRIP), is expressed specifically in rod and cone photoreceptors. It consists of an N-terminal region predicted to form coiled coil structures linked to a C-terminal tail that binds RPGR. In vivo, both proteins co-localize in the photoreceptor connecting cilia. RPGRIP is stably associated with the ciliary axoneme independent of RPGR and is resistant to extraction under conditions that partially solubilized other cytoskeletal components. When over-expressed in heterologous cell lines, RPGRIP appears in insoluble punctate and filamentous structures. These data suggest that RPGRIP is a structural component of the ciliary axoneme, and one of its functions is to anchor RPGR within the cilium. RPGRIP is the only protein known to localize specifically in the photoreceptor connecting cilium. As such, it is a candidate gene for human photoreceptor disease. The tissue-specific expression of RPGRIP explains why mutations in the ubiquitously expressed RPGR confer a photoreceptor-specific phenotype.

Genetic defects underlying RP1 are heterogeneous. The X-linked RPGR gene (1, 2) is clinically important because of the greater severity of disease and the higher percentage of patients associated with mutations in this gene (3). Neither the disease mechanism nor the physiological function of RPGR is fully understood. Analyses of the RPGR protein sequence, however, provide some clues. The N-terminal domain of RPGR shares sequence similarity with the regulator of chromatin condensation (RCC1), a nuclear protein that catalyzes guanine nucleotide exchange for the small GTPase Ran. RCC1 has an essential role in regulating nuclear import and export (4) by catalyzing the GTP exchange reaction for Ran. The presence of an RCC1 homology domain thus raises the possibility that RPGR may also regulate intracellular transport in photoreceptors via an unknown G protein.

RPGR is ubiquitously expressed. In photoreceptor cells, however, it is concentrated in the connecting cilium (5). We have previously studied the retinal phenotype of RPGR knockout mice (5). Photoreceptor defects are detected at an early age in mice lacking RPGR. Cone photoreceptors exhibit ectopic localization of cone opsins (the photopigment in cones similar to rhodopsin in rods) in the cell body and synapse. Although ectopic distribution of rhodopsin is not apparent in rods by immunofluorescence, there is nevertheless a reduced level of rhodopsin in the outer segments. Subsequently, both cone and rod photoreceptors degenerate. These data are consistent with the proposal that RPGR plays a role in maintaining the polarized protein distribution across the connecting cilium by facilitating directional transport or restricting redistribution (5).

To investigate the in vivo function of RPGR and the disease mechanism associated with RPGR mutations, we used a yeast two-hybrid screen to identify potential interacting partners of RPGR. This effort led to the discovery of a novel protein that interacts with the RCC1 domain of RPGR. After substantial completion of this work, two reports (6, 7) were published describing the identification of a bovine and human RPGR interacting protein. Comparison of the sequences shows that the protein we have identified is the murine ortholog of the bovine and human RPGR-interacting protein. Adopting their designation, we refer to this protein in the present report as RPGRIP (for RPGR interacting protein). We further demonstrate that RPGRIP is a structural component of the ciliary axoneme. We propose that RPGRIP serves as a scaffold to anchor regulatory complexes including RPGR within the connecting cilium.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—A GAL4-based two-hybrid system was employed. Cloning vectors, yeast host cells, and reagents were purchased from CLONTECH Laboratory (Palo Alto, CA). A retinal cDNA library was constructed using poly(A) RNA from C57BL/6 mouse retinas. The cDNAs were inserted into the pACT2 plasmid vector downstream of the GAL4 activation domain. The resulting cDNA library contained ~1 × 10⁶ independent colonies. The bait plasmid was constructed by inserting a cDNA encoding the bait protein into the pG-
BK7 plasmid vector downstream from the GAL4 DNA binding domain. The bait protein consisted of the RCC1 homology domain of murine RPGR (residues 39–460). Reference to the numbering of the RPGR sequence in this report is based on a previously published sequence (GenBank™ accession number AAC40190) (6). A sequential transient expression assay was used in this study. HeLa cells harvested from primary cultures were plated in 6-well tissue culture dishes, and cell lines were grown on coverslips in methanol at –20 °C for 5 min, washed with primary and Cy3-conjugated secondary antibodies. After the final wash, slides were stained with the nuclear dye Hoechst 33342 and mounted in aqueous mounting media. Dissociated photoreceptor cells were prepared and stained as described (10). To perform GST pull-downs, a yeast expression vector was used that inserts the C-terminal 253 amino acids of mouse RPGR (RPGR-253) was described to the N-terminus with either a Myc or an EGFP epitope. The GST moiety was removed from the glutathione-Sepharose beads were washed three times in TTBS, grids were incubated with goat anti-rabbit secondary antibody conjugated to 5 nm gold and washed again. To perform silver enhancement, a goat anti-rabbit secondary antibody conjugated to 1 nm gold particles was used instead. Sections were then treated with the Aurion R-Gent silver enhancement reagents (Electroscience Laboratories) for 8 min. Sections were post-stained with 4% uranyl acetate, washed through drops of methyl cellulose, and air dried. Sections were viewed and photographed on a JEOL 100CX electron microscope.

In Vitro GST Pull-downs and Co-immunoprecipitation— Purified GST-RPGRIP (residues 991–1331) fusion protein and the native RPGR present in mouse brain lysate were used in the pull-down assays. Mouse brain was mechanically homogenized in a lysis buffer (20 mM Tris, pH 7.4, 1% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (Roche Molecular Biochemicals)). The homogenate was cleared by centrifugation at 20,000 × g for 30 min. GST-RPGRIP or GST (negative control) coupled to glutathione-Sepharose was incubated with the indicated amounts of brain lysates adjusted to the same final volume in lysis buffer at 4 °C overnight. The glutathione-Sepharose beads were washed three times in TTBS, pH 7.4, 0.5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). Bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

Expression and Analyses of RPGRIP and RPGR in Cultured Cells— COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. Transfections were carried out using the Genebuckle 40 reagent (Quantum Biotechnologies) according to the manufacturer’s instructions. To express RPGRIP, three expression plasmids were constructed. The full-length coding region of RPGRIP was inserted into the pcDNA3 (Invitrogen) to generate the pRPGRIP plasmid for expression of untagged RPGRIP. To generate recombinant RPGRIP that were tagged at the N-terminus with either a Myc or an EGFP epitope, coding sequences for myc or EGFP were inserted into pBHRIP upstream and in frame with the RPGRIP coding sequence, generating plasmids pMyc-RPGRIP and pEGFP-RPGRIP, respectively. For co-immunoprecipitation of recombinant RPGRIPs, COS cells were co-transfected with pmyc-RPGRIP and pEGFP-RPGRIP. After 48 h, cells were washed with PBS and lysed in an immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and a protease inhibitor mixture). The lysate was centrifuged at 16,000 × g for 15 min. Immunoblot analyses indicated that about 1–5% of the total RPGRIP expression in COS cells was recovered in this supernatant. Immunoprecipitation was allowed to proceed overnight at 4 °C by incubating the cleared lysate with an anti-Myc monoclonal antibody (clone 9E10, Calbiochem) or an anti-EGFP polyclonal antibody (Santa Cruz) and protein A–agarose. After washing, bound proteins were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE, and detected with immunoblotting with the anti-RPGRIP antibody. For chemical cross-linking, cells transiently transfected with pRPGRIP were washed with PBS and incubated with the indicated amount (Fig. 2B) of dimethyl pimelidate (DMP) in 100 mM HEPES buffer (pH 8.2) for 1 h at 4 °C. The reaction was terminated with a
quenching buffer (100 mM Tris, pH 7.4, in PBS). After removal of the quenching buffer, the cells were lysed in SDS-PAGE sample buffer.

**Cellular Fractionation and Extractions**—The procedure consisted of collecting and extracting the inner and outer segment enriched fraction produced by shaking freshly dissected retinas in a homogenization buffer (11). Under Nomarski optics, these preparations were seen to contain outer segments linked to the connecting cilia with variable portions of inner segment membranes attached (10). The remainder of the retinas usually remained as intact sheets, thus providing an enrichment of photoreceptor-derived material in the suspension. Unless otherwise noted, tissues were kept on ice or at 4 °C during the procedure. In a typical experiment, 16 freshly dissected mouse retinas were vortexed for 1 min in 0.6 ml of homogenization buffer (34% sucrose, 25 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10 mM iodoacetamide, and a mixture of protease inhibitors). The suspension was centrifuged at 500 × g for 2 min to remove large debris. The supernatant was diluted 1:3 with a dilution buffer (homogenization buffer without sucrose) and centrifuged again at 170,000 × g for 15 min. The supernatant was removed to fresh tubes and was designated the cytosol fraction. The pellet was resuspended in the dilution buffer containing 1% Nonidet P-40. Aliquots of 100 μl each were spun at 170,000 × g for 20 min. The supernatant was pooled and designated as the detergent-soluble fraction and the pellets the axoneme-enriched fraction. Although the scarcity of mouse retinal tissues precluded the use of sucrose gradient centrifugation, which would have yielded a cleaner axonemal preparation (12), the enrichment of α- and acetylated α-tubulins and tektin filaments in our preparation validates its designation as axoneme-enriched (see Fig. 7). Pellets were extracted for 30 min at room temperature in the dilution buffer supplemented with one of the following reagents: 0.5% SDS, 1 M NaCl, 6 M urea, 0.5% saponin, 10 mM MgATP, or 2.5 mM AMP-PNP. Samples were centrifuged again at 170,000 × g for 30 min, and the supernatants and pellets were analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

**Identification of RPGRIP**—To identify interacting proteins, we performed a yeast two-hybrid screening of a mouse retinal cDNA library using the RCC1 homology domain of RPGR as bait. From among 106 co-transformants, six independent clones were isolated. Sequence analysis revealed that one clone coded for the d subunit of rod cGMP phosphodiesterase (PDEd), which had previously been reported (13). The remaining clones contained overlapping cDNAs apparently derived from the same gene. To obtain the full-length coding sequence, we screened a second mouse retinal cDNA library constructed in a λ phage vector using one of the original cDNA isolates as a probe. The longest among the second set of cDNA clones contained an open reading frame of 1331 amino acid codons (Fig. 1). The conceptually translated polypeptide has a predicted molecular weight of 152 kDa, is rich in glutamic acid residues, resulting in a rather acidic isoelectric point (pI 4.8), and is highly hydrophilic with no predicted membrane spanning regions. This protein is referred to as RPGRIP, for RPGR-interacting protein, following the designation given in two recent reports (6, 7). Search of the database identified a BAC clone (GenBank™ accession no. Al135744) that encodes the human ortholog of RPGRIP. This places the human RPGRIP on chromosome 14q11. The next closely related sequence to RPGRIP in the database is a human gene of unknown function (KIAA1005; GenBank™ accession no. AB023222). A comparison of the overlapping cDNA clones from the initial yeast two-hybrid screen localized the RPGR-binding domain within the C-termin-
The 1331-residue open reading frame transiently expressed in COS cells (lane 1) has the same apparent molecular mass as the native RPGRIP from retina (lane 2). B. GST pull-down assay for RPGR and RPGRIP binding. The GST-RPGRIP fusion protein was incubated with varying amounts of mouse brain lysate (as a source for native RPGR) followed by pull-down with glutathione-Sepharose. Bound proteins were analyzed by immunoblotting. The first lane on the left shows the total amount of RPGR present in 10 μl of brain lysate (loaded on the gel directly). The last lane shows a negative control in which a GST protein, instead of the GST-RPGRIP fusion protein, was incubated with the brain lysate.

To confirm that the coding sequence of RPGRIP was full-length at the 5' end, the 5' RACE procedure was performed. Sequences of the RACE products provided an additional 30 base pairs at the 5' end but did not further extend the open reading frame. Subsequent immunoblotting analyses showed that the native RPGRIP from mouse retina and a recombinant protein from transfected COS cells expressing the 1331 open reading frame had the same apparent molecular weight on SDS-PAGE (Fig. 2A), lending further support to the claim that this open reading frame is full-length. The apparent molecular mass of RPGRIP is 210 kDa, larger than the predicted 150 kDa. The reduced motility of the polypeptide on SDS-PAGE may be accounted for by the high glutamic acid content (14%) in this protein.

Confirmation of a direct interaction between RPGR and RPGRIP was provided by an in vitro GST pull-down assay. A GST-RPGRIP fusion protein containing the RPGR binding domain was shown to co-sediment with RPGR from tissue lysate in the presence of glutathione-Sepharose (Fig. 2B). Therefore, the recombinant RPGRIP interacts with native RPGR in vitro. A demonstration of the interaction between two native proteins by co-immunoprecipitation was not technically feasible because RPGRIP could not be solubilized under native conditions (see below).

Tissue-specific Expression of RPGRIP—The tissue expression pattern of RPGRIP was examined by Northern blotting (Fig. 3A) and immunoblotting (Fig. 3B). RNAs from a number of mouse tissues were examined. Initially a double-stranded DNA probe P1 (see bottom of Fig. 1) revealed a doublet band around 10 kb in the retina only and a 4.4-kb band that is more widely distributed. To determine which band(s) represented the authentic RPGRIP transcript, three additional probes were synthesized and used to probe Northern blots. Probe P2 was located upstream from P1 and entirely within the coding region. Probe P2 detected the 10-kb doublet but not the 4.4-kb band. Furthermore, the 10-kb doublet was present only in the wild type retina but not in the adult rd mouse retina in which the photoreceptors had degenerated, indicating a photoreceptor-specific origin of these transcripts. Thus, if the 4.4-kb band represented an RPGRIP transcript, it would have only the capacity to encode a protein comprising essentially the RPGR binding domain alone. Existence of such a variant would have functional implications. Alternatively, this band might be derived from an overlapping but otherwise unrelated gene. A data base search indicated that the gene for a chromatin-specific transcription elongation factor (16) overlaps with RPGRIP at the 3' end and is transcribed in the opposite orientation. To explore the latter possibility, probes P3 and P4, covering the same region as P1 but synthesized as single-stranded RNA probes in the antisense (P3) or sense (P4) orientation, were used to probe Northern blots. P3 detected only the 10-kb doublet, whereas P4 detected only the 4.4-kb band. This finding confirmed that the 4.4-kb band was derived from an overlapping gene transcribed in the reverse orientation. Therefore the 10-kb doublet represents the authentic RPGRIP transcripts, and both should encode the full-length RPGRIP protein. The size difference is likely to reside in the 3' untranslated region because of alternative use of transcription termination signals. Finally, probe P2 detected a much smaller transcript (3 kb) found only in the testis. The functional significance of this variant, if any, is not clear. It should be noted that the testis is thought to express irrelevant transcripts which, in somatic tissues, may be tightly regulated (17).

Immunoblotting of RPGRIP protein indicates a similar pho-
to receptor-specific expression (Fig. 3B). A polyclonal antibody raised against the RPGR binding domain of RPGRIP detects the 210-kDa polypeptide in the retina but not in a variety of other tissues. The RPGRIP band is also present in the retinas of RPGR knockout mice.

**RPGRIP Is Localized in the Photoreceptor Connecting Cilium**—Photoreceptors are highly polarized sensory neurons with their outer segments, inner segments, nuclei, and synapses organized into distinct layers on retinal sections, which makes it relatively simple to determine the subcellular localization of a protein on retinal sections. By immunofluorescence microscopy, we previously determined that RPGR is localized in the connecting cilia of rod and cone photoreceptors (5). To determine the subcellular location of RPGRIP in photoreceptor cells, we performed immunostaining of retinal sections using the polyclonal RPGRIP antibody. The data show RPGRIP is located at the junction between the inner and outer segments (Fig. 4A), suggesting a localization in the connecting cilia. This staining pattern is identical to that of RPGR (Fig. 4A). The RPGRIP localization remains the same in the absence of RPGR (Fig. 4A). This would suggest that RPGRIP is independently targeted to the connecting cilium, whereas RPGR is anchored to this location via its interaction with RPGRIP. Immunostaining of frozen sections of the lungs indicated that RPGRIP is not present in the motile cilium of airway epithelia (not shown).

Photoreceptor connecting cilium originates from a pair of basal bodies, located at the apical inner segment and related structurally to centrioles. The axonemal microtubules grow out of one of the basal bodies and continue into the outer segment (18). To determine whether RPGRIP is confined to the connecting cilium, or whether it extends proximally to the basal body and distally into the outer segment, we performed double labeling for RPGRIP and γ-tubulin, a marker for the basal body (10), on photoreceptors that had been mechanically dissociated. These preparations contained mostly shaken-off rod outer segments attached to the connecting cilia. Comparison of the immunofluorescence and Nomarski images indicated that RPGRIP localization is well defined; staining is limited to the connecting cilium and ends abruptly at the junction where the cilium joins the outer segment. Only trace amount of staining appear to be in the basal bodies (Fig. 4B). A schematic diagram of a photoreceptor cell is shown in Fig. 4C to help illustrate the subcellular localization pattern. The staining pattern of RPGRIP strongly resembles that of RPGR in dissociated photoreceptor cells (5).

Because mouse photoreceptors are overwhelmingly rods, the data described above provided no evidence concerning the localization of RPGRIP in cones or whether it is present in cones at all. To address this question, we performed immunostaining for RPGRIP on the cone-dominant retinas of 13-lined ground squirrels. In contrast to mice, ground squirrel retinas are ~95% cones (19). On retinal sections, immunostaining with the RPGRIP antibody prominently labeled the connecting cilia in this cone-dominant retina (Fig. 4D). The RPGRIP antibody, although generated with a murine antigen, recognizes a region in RPGRIP that is highly conserved among the mammalian species, suggesting that immunostaining in the ground squirrel was likely to be valid. These data demonstrate that RPGRIP is localized in the connecting cilia of both rod and cone photoreceptors.

Immunogold labeling and electron microscopy corroborated the findings by immunofluorescence. An examination of multiple sections showed that all the connecting cilium profiles are labeled with little background staining in the inner and outer segments. As shown in Fig. 5, labeling was restricted to the connecting cilia but was not found in the basal body. In the transverse view of connecting cilia, the 5-nm gold particles, on average, do not overlap with the profiles of the microtubule doublets. They appear on the external side of the ring of microtubule doublets. In this regard, the ultrastructural labeling of RPGRIP is somewhat similar to that of myosin VIIa (20). An examination of longitudinal sections revealed a similar finding (not shown).

**Expression of RPGRIP in Heterologous Cell Lines**—The essence of the coiled coil domain in RPGRIP suggests that it may normally exist as dimers or higher order polymers. To examine the state of existence of RPGRIP, recombinant RPGRIP, either tagged or untagged, was expressed in COS cells. By immunofluorescence or by following the green fluorescence protein tag, RPGRIP in transfected cells was seen in punctate and filamentous patterns (Fig. 6A). Double labeling experiments showed that RPGRIP did not co-localize with microtubules or several intermediate filament proteins including vimentin and cytokeratins. Furthermore, the filamentous staining pattern of RPGRIP was preserved in cells in which the microtubules had been disrupted by treatment with nocodazole (data not shown). These data suggest that RPGRIP may exist in large polymers in the cultured cells. Consistent with this notion, cellular fractionation and SDS-PAGE analyses showed less than 5% of RPGRIP could be recovered in the supernatant; the remainder was present in the detergent-insoluble pellet. Limited cross-linking produced higher molecular weight species consistent with dimer and higher order oligomers (Fig. 6B). As a second test for oligomer formation, COS cells were co-transfected with plasmids carrying c-Myc- or EGFP-tagged RPGRIP. The cell lysate was then immunoprecipitated with either a c-myc or an EGFP antibody. As shown in Fig. 6C, either antibody was able to co-precipitate both recombinant proteins, providing further evidence for homodimer or higher oligomer formation by RPGRIP in the transfected cells. Taken together, these data suggest that RPGRIP polymerizes into a higher molecular weight complex in transfected cells.

**RPGRIP Is a Structural Component of the Ciliary Axoneme**—To examine the nature of its association with cilium in greater detail, a series of cellular fractionation and extraction experiments were carried out. In these experiments, photoreceptor inner and outer segments were shaken off, collected and extracted with detergent or high salt. As shown in Fig. 7A, RPGRIP was not detectable in the cytosol, nor could it be solubilized by treatment with the detergent Nonidet P-40. It was found exclusively in the detergent-insoluble fraction enriched for ciliary axoneme. Treatment with saponin, or with ATP and its nonhydrolyzable analog AMP-PNP, produced identical results (not shown), indicating that RPGRIP was not associated with detergent-insoluble lipids (21) or indirectly tethered to the axoneme via an association with kinesins. Thus, RPGRIP is likely to be directly associated with, or is a part of, the axonemal cytoskeleton. This is in contrast to RPGR, which exhibited a sizable cytosolic pool in addition to the fraction associated with the cilium. Considering the cilium is such a minute structure relative to the inner and outer segments, the data is fully consistent with our observation that RPGR is concentrated in the connecting cilium.

The detergent-insoluble fraction from above was further extracted with high salt or denaturants. The results are shown in Fig. 7B. RPGRIP was completely resistant to extraction by high salt and was only solubilized by the strong denaturants, SDS and urea. In contrast, a substantial fraction of RPGR could be solubilized by high salt extraction, which is consistent with the suggestion that RPGRIP is specifically targeted to the cilium while RPGR is recruited to this cellular compartment as a secondary event. We then compared this extraction profile with
that of ciliary cytoskeletons and tektin filament. Ciliary microtubules are of the stable, acetylated form (22), and microtubules in the basal bodies contain γ-tubulin. Tektins are present as a stable protofilament in the basal bodies as well as in cilia and flagella and are thought to be integral to the outer axonemal microtubule doublets (23–25). Using specific antibodies for these proteins, to examine the different fractions from the same experiments, we found that all were partially solubilized by high salt treatment. Thus the RPGRIP complexes appear to be less soluble. The axoneme-enriched fraction was also sub-

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**Fig. 4. Immunofluorescence microscopy.** A, retinal sections stained with antibodies for RPGRIP (left) or RPGR (right). Genotypes of mice are indicated. RPGRIP staining is similar in both genotypes, but RPGR staining is abolished in the RPGR knockout mouse. RPE, retinal pigment epithelium; OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer; WT, wild type. B, staining for RPGRIP in dissociated rod photoreceptors. DIC, differential interference contrast optics. RPGRIP staining is largely confined to the cilium (black arrowheads). γ-Tubulin staining indicates the position of the basal bodies (white arrowheads). C, schematic diagram of a rod photoreceptor. D, staining for RPGRIP in the cone-dominant ground squirrel retina indicates localization of RPGRIP in the connecting cilia of cones as well. An arrowhead indicates the position of CC.
RPGR-interacting Protein Is Associated with the Cilium

Fig. 5. Ultrastructural localization of RPGRIP in transverse sections. RPGRIP in the connecting cilium (CC) is revealed by the 5-nm gold particles, which appear external to the profiles of the nine microtubule doublets. Left, two photoreceptor profiles are shown. The upper one was sectioned at the plane of the connecting cilium and the apical inner segment of the same cell. The lower one was sectioned at the level of the basal body (BB), as indicated by its enclosure within the inner segment. Note lack of labeling in the basal body. Upper right, a profile of connecting cilium at a higher magnification. Lower right, a profile of connecting cilium with silver enhancement following immunogold labeling. M, mitochondria; IS, inner segment. Bar = 0.1 μm.

DISCUSSION

Although the importance of RPGR in the pathogenesis of RP is now recognized (3), the physiological function of RPGR and the molecular basis of disease caused by its mutations remain elusive. To date, few reports have appeared on the analyses of the native RPGR protein from the retina. In a previous study, we found RPGR to be concentrated in the connecting cilia of rod and cone photoreceptors. Furthermore, analyses of mice lacking RPGR indicated a role for RPGR in maintaining the polarized distribution of proteins across the connecting cilia (5). In an attempt to delineate the biochemical pathway in which RPGR is a component, we used a protein interaction screen to identify proteins that may physiologically interact with RPGR. With the RCC1 homology domain of RPGR as a bait, we have isolated an interacting protein, RPGRIP, and demonstrated its co-localization with RPGR in the connecting cilium. We found a significant difference between RPGR and RPGRIP in their association with the cilium. RPGR has both a ciliary pool and a sizable cytosolic (or loosely associated) pool. In contrast, RPGRIP is exclusively and stably associated with the ciliary axoneme. Because its ciliary localization is unchanged in RPGR knockout mice and because it remains bound to the ciliary axoneme when RPGR is fully solubilized, we conclude that RPGRIP is targeted independently to the cilium, whereas RPGR is anchored here through binding to RPGRIP. Given that the connecting cilium is a minute structure with a limited number of resident proteins, their co-localization in this cellular compartment and the finding that the expression of RPGRIP is specific to the photoreceptors provide strong evidence for the functional significance of their interaction.

The RPGRIP described here is the murine ortholog of the human and bovine RPGRIP proteins reported recently (6, 7). Differences between our work and the two reports are noted in several aspects. First, the reported human sequences are shorter by up to 700 residues compared with the murine sequence reported here. Analysis of the human RPGRIP genomic sequence in GenBankTM with the GenScan program predicted 5′ coding exons that are not included in the two reports (6, 7) that are highly homologous with the murine RPGRIP. These putative upstream exons could be identified by reverse transcription-polymerase chain reaction using human retinal mRNA as a template.3 Therefore, the reported human RPGRIP sequences may not be complete at the 5′ end. Second, we found no evidence in murine tissues for substantial alternative processing in the coding region of the RPGRIP transcript. We examined such a possibility extensively with Northern blot analysis. Initial Northern blotting data indicated the possible presence of a variant encoding a much smaller protein; this raised the interesting possibility that a soluble RPGR interacting protein (without the coiled coil domain) could sequester RPGR in a different cellular compartment, which would have functional implications. Further examination with single-stranded probes indicated that the smaller transcript is derived from an overlapping gene. Data base search also indicates that this overlapping gene organization is conserved between the mouse and human genomes. Third, one of the reports found co-localization of RPGR and RPGRIP in a different subcellular compartment, i.e. the outer segment (7), whereas we have

3 D.-H. Hong, G. Yue, M. Adamian, and T. Li, our unpublished observation.
concluded that RPGRIP localizes exclusively in the connecting cilium and is presumably responsible for concentrating RPGR in the cilium. The reason for this discrepancy is not yet clear, but we suggest it could be accounted for by the differences in the procedure for tissue fixation. In particular, we observed that aldehyde fixation prior to embedding and sectioning was detrimental for staining both RPGRIP and RPGR at the light microscopy level. This appears to limit access by the antibodies rather than destroy the antigenic epitopes, because dissociated photoreceptors which would have lost much of the materials surrounding the cilia are much less sensitive to aldehyde fixation. Ultrathin frozen sections, which would have antigenic sites more fully exposed, could also withstand pre-embedding fixation.

The photoreceptor is a highly polarized cell in which a non-motile connecting cilium links two morphologically and functionally distinct parts, the inner and outer segment. The ciliary axoneme originates from a basal body located at the distal end of the inner segment, and its core structure is composed of nine outer microtubule doublets but no central microtubule singlet, forming what is usually referred to as a 9 + 0 arrangement. The structural organization is analogous to the transition zone of motile cilia (26, 27). Unlike motile cilia, however, the connecting cilium daily transports a prodigious amount of proteins...
including rhodopsin (see Ref. 28 for a recent review) and other components of phototransduction. The inner segment contains all of the organelles for biosynthetic and metabolic functions. The outer segment is a specialized organelle where the phototransduction cascade takes place. The outer segment is continually renewed throughout life by shedding older portions at the tip and adding new membranes at the base. As the sole physical link between the inner and outer segments, the connecting cilium is critically important for the directional transport of nascent proteins destined to the outer segment, against a steep concentration gradient. Because the inner and outer segments have very different protein composition, the connecting cilium also acts as a diffusion barrier against redistribution (29). Maintenance of the outer segment is essential for efficient phototransduction, as well as for the long term survival of the photoreceptor cell as demonstrated by studies in animal models. Interestingly, a number of proteins also exhibit light-dependent rapid movement across the cilium, although its physiological significance remain undefined (30). Thus the finding that RPGRIP anchors RPGR in the connecting cilium is consistent with their putative function in regulating protein transport in the connecting cilium.

In transfected COS cells, RPGRIP exhibits a mixed punctate and filamentous pattern. This does not appear to result from binding to an existing cytoskeletal structure because in double labeling experiments RPGRIP staining does not fully overlap with microtubule or intermediate filaments. These data indicate that RPGRIP expressed in cultured cells polymerizes into high molecular weight complexes that are resistant to detergent solubilization. The coiled coil domain is likely to play a part in mediating the oligomer formation.

In the cilium, RPGRIP also appears to exist in higher molecular weight complexes. Judging by data from chemical cross-linking and electron microscopy, the RPGRIP complex does not appear to be in extensive contact with or integrated into the microtubule arrays. Two possibilities can be envisioned. RPGRIP may form filamentous structures running parallel to the axonemal microtubules, although no ultrastructural evidence currently exists for such a structure. Alternatively, RPGRIP may be a component of the microtubule-membrane cross-linkers. These are the Y-shaped structures seen in transverse sections that project from each microtubule doublet at the junction between the A and B tubules to the adjacent plasma membrane (12, 31). These structures co-purify with the ciliary axoneme and are resistant to detergent extraction. Their location is confined to the connecting cilium, matching that of RPGRIP. One caveat to this hypothesis is that a morphologically equivalent structure is also present in the transition zone of all motile cilia (32), and our data indicate that RPGRIP expression is confined to photoreceptors. These differences could be reconciled if related protein(s) are responsible for reconstituting the cross-linkers in motile cilia. Although its exact structural organization remains undefined, the localization of RPGRIP between the external wall of the microtubule doublet and the plasma membrane that surrounds it should bring it into close contact with soluble and membrane-bound proteins trafficking through or along the connecting cilium, either by intraflagellar transport or by movement along the plasma membrane (33–35). Thus, RPGRIP is well suited to serve as a scaffold to anchor RPGR and possibly other regulatory complexes, which in turn would control the directional transport of proteins to or from the outer segment.

Acknowledgment—We thank Michael Scimeca for technical assistance, Dr. Changwan Lu for constructing the cDNA libraries, and Drs. Virgil Muresan and Dorothy Roof for discussion.

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