RPGRIP1s with distinct neuronal localization and biochemical properties associate selectively with RanBP2 in amacrine neurons

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RPGR and RPGRIP1 are molecular partners with vital roles in retinal function. Mutations in RPGR are implicated in heterogeneous retinal phenotypes, while those in RPGRIP1 lead to Leber congenital amaurosis. RPGR and RPGRIP1s differentially localize in photoreceptors among species. This may contribute to phenotype disparities among species bearing mutations in RPGR. However, it cannot account for the phenotype heterogeneity associated with RPGR- and RPGRIP1-linked mutations in the human. The existence of RPGRIP1 isoforms with distinct cellular, subcellular localizations and biochemical properties in the retina is shown. High mass RPGRIP1 isoforms, p175/p150, enriched in the outer segment (OS) compartment of photoreceptors are identified. The remaining isoforms are present across subcellular fractions, including nuclei and are soluble. The p175/p150 are predominantly sequestered in the cytoskeleton-insoluble fraction of OS and nuclei. In selective amacrine cells, and in the transformed photoreceptor line, 661W, RPGRIP1s localize at restricted foci to nuclear pore complexes and/or the vicinity of these. Among the nucleoporins, RPGRIP1 isoforms selectively associate in vivo with RanBP2 (Nup358). RPGRIP1s also decorate microtubules in 661W cells and occasionally form coiled-like inclusion bodies in the perikarya. These results support distinct but complementary functions of RPGRIP1 isoforms in cytoskeletal-mediated processes in photoreceptors and amacrine neurons, and may explain the Leber phenotype linked to RPGRIP1 mutations in humans. Moreover, the data implicate a role of RanBP2 in the pathogenesis of neuro(retino)pathies and as a docking station to mediate the nucleocytoplasmic shuttling of RPGRIP1s and their interaction with other partners in amacrine and 661W neurons.

INTRODUCTION

The RPGR gene encodes several alternative spliced isoforms of the ubiquitously expressed retinitis pigmentosa GTPase regulator (1–4). In the human, mutations in RPGR have been shown to lead to a host of severe X-linked retinal dystrophies such as retinitis pigmentosa (RP) (1,4–6), cone–rod (7) and cone (8) dystrophies, and recessive atrophic macular degeneration (9). All missense mutations identified in RPGR until this date are within its RCC1-homologous domain (10), which is strictly conserved among all RPGR isoforms and encoded by the first 11 of the 19 exons comprising the RPGR gene (1,11). Recently, an RPGR isoform, RPGR_{ORF15}, was identified (4). This isoform is generated through the skipping of the cryptic acceptor splice site of exon 15 and consequent translation of part of intron 15 (ORF15) (4). The extended RPGR_{ORF15} contains a novel C-terminus highly enriched in glutamate/aspartate and glycine repeats. The ORF15 is a mutational hot spot and accounts for close to 70% of all XIRP3 mutations in the human. All mutations in the human ORF15 consist of frame-shift and nonsense mutations and lead to heterogeneous retinal dysplasias (4,7–9). Similar mutations have been identified in the XIRP3-affected dogs (12). These genetic data underscore a critical role of the RCC1-homologous domain and of that encoded by the ORF15 in RPGR function in the retina.

The complexity of heterogeneity of RPGR transcripts, mutations and phenotypes in the human was compounded with the observation of phenotype disparities among certain species. Targeted deletion of all RPGR isoforms in the mouse leads to mild retinal disease phenotypes (13). In contrast,
XIRP3 mutations in the human and the dog lead to extremely severe phenotypes (12,14–16). Recently, we proposed that the restricted localization of RPGR isoforms in the connecting cilium/axoneme of the mouse (13,17), in contrast to their broader localization in the outer segments of cone and rod photoreceptors of the human and bovine (17,18), may account for the differences in the expression of the disease among species (17). This differential expression may also reflect differences in the cytoarchitectural organization of the OS among species (17).

To determine the molecular basis of the retina-specific pathogenesis of mutations in RPGR, we isolated RPGR-interacting protein 1 isoforms (RPGRIP1s) (18,19). All RPGRIP1s isolated are derived by alternative splicing (18,19). Likewise, other laboratories isolated similar RPGR substrates (20,21). RPGR interacts directly in vivo and in vitro, with the RPGR-interacting domain (RID) at the C-terminus of RPGRIP1 and they colocalize in the outer segment of photoreceptors in the bovine and the human (18). Missense mutations in RPGR severely uncouple in vivo its interaction with RPGRIP1 (18). This supports mutations in RPGR result in the uncoupling of RPGR with RPGRIP1 and, thus, the loss-of-function of these. Subsequently, human mutations in RPGRIP1 were discovered to lead to Leber congenital amaurosis (LCA) (22,23), which is clinically distinct from RP. LCA is the most severe form of all retinal dysplasias and with the earliest onset from birth. The molecular basis for the extreme severity of LCA caused by mutations in RPGRIP1 (and other genes) is not understood. However, children displaying LCA often exhibit severely diminished or absent electroretinograms (ERG) accompanied by other ocular phenotypes (24–26). This suggests that LCA-causing genes may play important roles in inner retinal neurons in addition to that in photoreceptors. We previously reported strong expression of RPGRIP1 in amacrine cells of bovine retinas (17). This may provide a rationale for the discrepant phenotypes caused by mutations in RPGR and RPGRIP1 in light of the role of these cells in retinal development and in coupling excitatory/inhibitory pathways between cone/rod bipolar cells and ganglion cells (27).

To determine the molecular basis of LCA caused by RPGRIP1 mutations and understand RPGRIP1 function, we report here distinct subcellular and biochemical profiles between RPGRIP1s expressed in photoreceptors and other retinal neurons. We provide evidence of RPGRIP1 association in vivo with RanBP2 at the nuclear rim of a restricted population of amacrine neurons and in the transformed murine photoreceptor line, 661W.

RESULTS

RPGRIP1s colocalize with nucleoporins at discrete foci in the nuclear rims of amacrine cells

Recently, we localized RPGRIP1 to the outer segment (OS) of photoreceptors of bovine and human retinas, while in murine photoreceptors they were localized to the connecting cilium/axoneme (17). These experiments also hinted expression of RPGRIP1s in amacrine cells (17). To investigate the localization of RPGRIP1s in amacrine cells in detail, we carried out immunolocalization of RPGRIP1 on Triton-X100 permeabilized retinal sections of three species (Fig. 1) with antibodies against the C2 domain (protein kinase C conserved region 2) of RPGRIP1s (17,21) (Fig. 4A and B). These antibodies strongly stained a subset of neurons in the inner nuclear layer (INL) of the retina of bovine (Fig. 1A, arrows), human (Fig. 1B, arrows) and mouse (Fig. 1C, arrows). In the former species, the strongest expression RPGRIP1s in the INL was observed in amacrine cells located at the proximal edge of the INL (Fig. 1A, arrows), albeit other neurons of this layer also expressed RPGRIP1. In the INL of the human and the mouse, RPGRIP1 was restricted mainly to amacrine cells (Fig. 1A and B; arrows). In these cells, RPGRIP1 was mostly localized to the nuclear envelope and/or perinuclear region and axons (Fig. 1C, open arrowheads) emanating from the cell bodies. Such localization was confirmed with other antibodies against the RPGR-interacting domain (RID) of RPGRIP1 (Fig. 1E and data not shown). In addition, few neurons in the ganglion cell layer were also stained in all three species (Fig. 1A–C; solid arrowheads). The paucity of RPGRIP1-immunopositive neurons in this layer suggests that they represent displaced amacrine cells (27). In light of the extreme proximity of RPGRIP1 to the perinuclear region, we employed two monoclonal antibodies, mAb414, against the XAEXFG domain present in some nucleoporins (28–30) such as RanBP2, Nup153 and Nup62, and another against nuclear pore-O-linked glycoproteins (31,32), to determine whether RPGRIP1 colocalized with these subcellular markers. Nucleoporins are proteins often located at nuclear pore complexes and their vicinity. As shown in Figure 1F and G, RPGRIP1s often colocalized with the nucleoporin markers at very restricted foci around the nuclei of a subpopulation of amacrine cells in bovine and murine retinas.

Then we carried out high resolution and deconvolution epifluorescence microscopy of serial Z-stacks images to confirm and to determine precisely the localization of RPGRIP1 in amacrine cells, the nuclear envelope and/or its association with components of the nuclear pore complexes (NPCs; Fig. 2). These studies were carried out with murine retinas in light of the morphometry of their nuclei and presumably lower density of NPCs in the retinal neurons of these species compared with those observed in the bovine. RPGRIP1 could be observed at highly restricted foci along the axons (Fig. 2B and C, open arrowheads) and nuclear rims (Fig. 2B, D, H and L) of some amacrine cells. Some of the RPGRIP1 foci colocalized with nucleoporins at the nuclear rim and/or its vicinity (Fig. 2C, arrows). In some amacrine cells, RPGRIP1 interfaced with nucleoporins at the cytoplasmic face of the nuclear envelope (Fig. 2F, open arrowheads), colocalized perfectly with nucleoporins (Fig. 2F, open arrowheads), and/or intercalated perfectly between NPCs without colocalizing with these (Fig. 2F, solid arrowheads). Because nuclear pore-O-linked glycoproteins are common components of NPCs (31,32), we employed another antibody, mAb414, against highly restricted nucleoporins such as RanBP2/Nup358 (30,33,34) and Nup153 (29). The latter is a nucleoporin associated with NPCs at their intranuclear face (29) while the former been described as a nucleoporin, Nup358, which is localized at the tips of cytoplasmic filaments emanating from NPCs (34–36). RanBP2 is also highly abundant in neuroretinal cells (33,37), where it is
differentially localized to NPCs or their vicinity, and other subcellular compartments such as the ellipsoid of photoreceptors and cytoplasmic tracks of a subset of ganglion cells highly enriched in NPCs (37). As shown in Figure 2G–J, RPGRIP1 is juxtaposed with a restricted population of RanBP2/Nup153 immunopositive foci at the intranuclear (Fig. 2I and J, solid arrowheads) and cytoplasmic (Fig. 2I and J) faces of the NPCs of displaced amacrine cells located in the ganglion cell layer. In addition, other neurons in the INL of bovine retina (A and F) express RPGRIP1s (albeit at lower levels) and they may arise due to species-specific differences.

Figure 1. RPGRIP1s localize to a subset of amacrine cells in the retina across species and it colocalizes with nucleoporin markers. Radial cryosections of bovine (A), human (B) and mouse (C) retinas immunostained with MCW 3 antibody (10 µg/ml) against the C2 domain of RPGRIP1s (see Fig. 4B). RPGRIP1 was localized in inner retinal neurons of the INL (arrows), and sporadically of the GCL (solid arrowheads), across all three species. In particular, a subpopulation of amacrine cells located at the proximal edge of the INL (A, B and C, arrows) and displaced amacrine neurons in the GCL (solid arrowheads) expressed RPGRIP1 predominantly at their nuclear rims (or vicinity of these) (B, C; arrows) and axons (C; open arrowheads). In contrast to other species (A, B), RPGRIP1 was restricted to the connecting cilium in mouse photoreceptors (D). An antibody (Ab 39) against the conserved RID domain of RPGRIP1 (see Fig. 4B) localized RPGRIP1 to the nuclear rim and perikarya of amacrine cells (E). RPGRIP1 colocalized with nuclear markers at restricted foci in inner nuclear neurons of bovine (F) and murine (G) retinas (see also Fig. 2 for detailed colocalization analysis). (F) and (G), respectively, show localization of RPGRIP1 (red) and RanBP2/Nup153/Nup62 (green), and RPGRIP1 (red) and nuclear pore-O-linked glycoproteins (green), with MCW3 (10 µg/ml) and mAb414 (15 µg/ml), and MCW3 (10 µg/ml) and nuclear pore-O-linked glycoprotein (10 µg/ml) antibodies. The murine section in (E) is the same as in (C). Note that nuclear markers also stain other subcellular compartments. In addition, other neurons in the INL of bovine retina (A and F) express RPGRIP1s (albeit at lower levels) and they may arise due to species-specific differences. ROS, rod outer segments; RIS, rod inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer, INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer.

RPGRIP1 isoforms selectively form in vivo a complex with RanBP2

In light of the colocalization of RPGRIP1 with restricted NPC components and in particular, RanBP2/Nup153/Nup62, we employed coimmunoprecipitation assays with retinal extracts to investigate further whether RPGRIP1 associates in vivo and selectively with RanBP2, and to determine the identity of the RPGRIP1 isoforms associated with RanBP2. As shown in Figure 3A, western blot analysis of immunoprecipitation reactions with two independent antibodies against the Zn-finger (ZnF; lane 4) and kinesin-binding (KBD; lane 5) domains of RanBP2 (38,39) and the monoclonal antibody against RanBP2, Nup153 and Nup 62 (lane 6) (28–30), showed all three antibodies coprecipitated an RPGRIP1 isoform of 175 kDa. Interestingly, the antibody against kinesin-binding domain of RanBP2 precipitated a 175 kDa RPGRIP1 isoform in retinal extracts, suggesting that RPGRIP1 associates with RanBP2 in vivo in a complex with the kinesin-binding domain of RanBP2.
domain of RanBP2 (39) also coprecipitated an RPGRIP1 isoform of 95 kDa. These RPGRIP1 isoforms were neither precipitated with the protein A beads (lane 7) nor rabbit IgG (lane 8), while an antibody against the conserved RID domain of RPGRIP1 (control) (18) precipitated both isoforms (lane 3). Reciprocal coimmunoprecipitation with an anti-RPGRIP1 antibody confirmed the specific association of RanBP2 with RPGRIP1 (Fig. 3C, lane 2). To distinguish the association between RPGRIP1 and RanBP2 from those with other nucleoporins, which are part of the huge nuclear pore macromolecular complex, we carried out coimmunoprecipitations with an anti-RPGRIP1 antibody followed by western analysis with mAb414, which recognizes (as described) three topologically distinct nucleoporins, RanBP2/Nup358, Nup153 and Nup62. As shown in Figure 3D, the anti-RPGRIP1 antibody only coprecipitated specifically RanBP2 (lane 2) among the three and topologically distinct nucleoporins present in retinal extracts (lane 1).

**Distinct RPGRIP1s are differentially localized among retinal subcellular fractions and expressed among species**

The authors and others have previously found the RPGRIP1 gene is subjected to complex alternative splicing in the human
and bovine, albeit only a single transcript has been reported for the mouse (21) (Fig. 4). In light of these data (summarized in Fig. 4), localization of RPGRIP1s to the outer segment of photoreceptors (17,18) and nuclear rims of amacrine cells (Figs 1 and 2), and selective association of RanBP2 with RPGRIP1 isoforms (Fig. 3), we investigated whether there are RPGRIP1 isoforms differentially localized among retinal cells, subcellular compartments thereof, and species. To this end, we carried out immunochemical analysis of retinal homogenate extracts and subcellular fractions thereof with an antibody against the conserved RID domain of RPGRIP1 (Fig. 5A). We first focused on contrasting the RPGRIP1 isoforms present in the outer segment compartment of photoreceptors to those of retinas depleted of rod outer segments. We identified six RPGRIP1 isoforms in retinal homogenates with various molecular masses such as p175, p150, p95, p55, p45 and p33 (Figs 5A and 6). Some of these masses matched closely with those predicted from the translation products of transcripts isolated (Fig. 4B). For example, the predicted masses of bRPGRIP1a, bRPGRIP1b and hRPGRIP1d match closely that observed for p95 in the human and the bovine (Figs 4B and 5A). Likewise, the mRPGRIP1 and hRPGRIP1 (and bRPGRIP1) match closely p150 (Figs 5A, 6A and B). The human p150 has a slightly faster electrophoretic mobility than the murine counterpart (Fig. 6B, compare lanes 3 and 4) consistent with its slightly lower predicted molecular mass (146.7 versus 152 kDa; Fig. 4B). The bovine p175 isoform, which seems unique to bovine (see data below on murine 661W cells), exhibits some mass disparity with the predicted mass of bRPGRIP1. Thus, it is possible that p175 is encoded by an alternative splice transcript not yet isolated and/or post-translational modifications.
may change its electrophoretic mobility. The same is true for the human p55, which closest match is hRPGRIP1c, and other RPGRIP1 isoforms observed (e.g. p45, p33). In addition, limited proteolysis may lead to mass changes in the generated RPGRIP1 products (see data below).

Among all RPGRIP1 isoforms identified, the p175 and p150 were highly enriched in the outer segment fraction of rod photoreceptors (Fig. 5A, lane 5) containing rhodopsin (Fig. 5B) but just traces of cone opsin (Fig. 5C) and the resident connecting cilium protein, myosin VIIa (Fig. 5D) (40). The remaining isoforms are present either in the inner retinal fraction or equally distributed in this and the OS fractions. Figure 5A also shows that p55 is only present in human retinas among all three species analyzed, while in the mouse, p45 and p33 (Figs 5A and 6) are the preponderant isoforms. Interestingly, the abundance of the high molecular mass RPGRIP1 isoform enriched in the OS of photoreceptors (e.g. p175/p150) seemed lower in murine retinas in comparison to that seen in bovine and human retinas (Fig. 5A), albeit such RPGRIP1 isoform could be clearly observed upon longer exposure of the blots (Fig. 6B). The diversity of RPGRIP1s among species is further displayed in Fig. 6. Intriguingly,
rodent (rat and mouse) species seem to express mostly the OS-enriched p150 isoform, which is clearly seen upon longer exposure of blots (Fig. 6B). Other highly abundant and closely related masses of RPGRIP1s of ~45 kDa can be seen in ocular tissues of retinas of mouse and other species. Interestingly, dog retinas expressed an RPGRIP1 isoform of high molecular mass ~160 kDa and the overall abundance of all RPGRIP1s matched closely those seen in rodents (Fig. 6A and B).

We next determined the expression of RPGRIP1 isoforms in nuclei and other subcellular compartments of retinal neurons. To this end, intact nuclei were isolated and highly purified on a sucrose cushion from other subcellular compartments (Fig. 7A). Electron microscopy analysis of the nuclei isolated confirmed the extremely high integrity and purity of the retinal nuclei (Fig. 7B). This was further confirmed by immunochromatography analysis of the nuclear fractions with antibodies against specific nuclear markers (e.g. lamin A/C and Nur77). The nuclei and other subcellular fractions were resolved on SDS–PAGE and analyzed by western blot with an antibody against the conserved RID domain of RPGRIP1s (18). As shown in Figure 7D, the p175 isoform was much less abundant in the nuclei (Fig. 7D, lanes 1 and 2) and other subcellular fractions derived from a heterogeneous population of neuroretinal neurons compared to that seen with the crude outer segment and enriched rhodopsin fraction (lane 3; Fig. 7D, lower panel) of photoreceptor neurons. The apparent low abundance of p175 in the nuclei fraction reflects the limited representation of RPGRIP1-expressing amacrine cells (Fig. 1) among all 29 subtypes of amacrine cells reported to exist (41) and the total population of nuclei of retinal neurons. To this end, amacrine neurons constitute about 10% (42) and 39% (43) of the total population of neurons in the retina and INL in the murine species, respectively, and these numbers vary significantly among species (41). P95 was present in all subcellular fractions except the microsomal/cytosolic fraction, while p45 was primarily distributed in all fractions but nuclei. p33 was present in all fractions analyzed.

**The RPGRIP1s exhibit distinct biochemical properties**

To confirm further the identity and enrichment of p175 in OS, we carried out western blot analysis of bovine retinal fractions (Fig. 8A) with two independent antibodies, Abs 23 and 22, against the unique coiled-coil SMC domain of bovine RPGRIP1 (Fig. 4A and B). The identity of p175 as RPGRIP1 was confirmed unequivocally by immunopurification
(data not shown) followed by the determination of its partial sequence by Edman degradation (Fig. 8B). As shown in Figure 8A, Ab 23 exclusively detects p175, which is enriched in OS (Fig. 8A, lane 4) but of low abundance in supernatants of NP-40 detergent solubilized extracts of OS (Fig. 8A, lane 5) and retinal extracts (Fig. 8A, lane 2). Likewise, Ab 22 exclusively detects p175 from bovine retinas (data not shown). The low abundance of p175 in detergent-solubilized extracts prompted us to investigate the solubility properties of RPGRIP1s as shown in Figure 8C. To this end, p175 was partially extracted from the OS compartment of photoreceptors with Nonidet P-40 (NP-40) (Fig. 8D, lane 2), but none of the remaining protein was extracted with Triton-X100 (Fig. 8D, lane 3) and the chaotropic agent, urea (Fig. 8D, lane 4). In contrast, the remaining RPGRIP1 isoforms were mostly extracted from the OS with NP-40 (Fig. 8D, lane 2) and Triton-X100 detergents (Fig. 8D, lane 3) although the p33 was partitioned among the detergent (Fig. 8D, lanes 2 and 3) and the urea (Fig. 8D, lane 4) fractions but not in the remaining SDS-solubilized pellet (Fig. 8D, lane 5). Interestingly, the p95 isoform is presumably produced by limited proteolysis upon detergent solubilization with NP-40 (Fig. 8D, lane 2), since it was not observed in the starting material (lane 1). Then, we employed similar extraction procedures (Fig. 8C) to investigate the biochemical solubility properties of RPGRIP1s derived from highly purified nuclei of retinal neurons (Fig. 8E). p175, p95 and p33 isoforms of RPGRIP1 were clearly observed in the initial preparation of nuclei (Fig. 8E, lane 1). Triton X-100 extraction solubilized completely p95 and a fraction of the p33 pool (Fig. 8E, lane 2). It also seemed to render the production of the p45 and p33 isoforms by limited proteolysis, as the former isoform is not observed in the starting nuclei material (Fig. 8E, lane 1) and the amounts of the latter increased significantly. Further extraction with urea led to the solubilization of most of the remaining p33 (Fig. 8E, lane 3). p175 remained in the insoluble, detergent and urea-extracted pellet (Fig. 8E, lane 4).
p175 is expressed in the transformed murine photoreceptor line, 661W, and RPGRIP1s colocalize with RanBP2/NPCs and microtubules at restricted foci

To help further delineate the role of RPGRIP1s in retinal neurons, we employed the transformed murine (cone) photoreceptor line, 661W, to characterize RPGRIP1 isoforms, their subcellular localization and contrast this to that observed for primary retinal neurons. This line expresses red opsin and other photoreceptor proteins (data not shown) (44–47). In addition to p150 and p45 observed in murine retinas (Fig. 6), the 661W cells expressed strongly two unique RPGRIP1 isoforms, p62 and p175, which are barely and not detected in murine retinas (Fig. 9A, e.g. compare lanes 1 and 2 with lane 3), respectively. Interestingly, the p175 isoform was only observed in primary neurons of bovine retina as previously noted. RanBP2, Nup153 and Nup62 expression in 661W cells is comparable to that seen in bovine retinal extracts (Figs 3D and 9B). Then we carried out colocalization studies of RPGRIP1s with NPCs and the cytoskeletal markers, tubulin and actin, because of the association of RPGRIP1s with retinal RanBP2 previously described and the presence of RPGRIP1 (p175/p150) in the insoluble cytoskeletal pellets of detergent-extracted nuclei and ROS. In 661W cells, RPGRIP1s are located clearly at the nuclear rim and/or its vicinity, intranuclear and dispersed throughout the perikarya in puncta and short filament-like tracks (Fig. 9D). In addition, RPGRIP1s could be observed in strongly stained coiled-like inclusion bodies of some cells (Fig. 9G). RPGRIP1s colocalized with a restricted population of NPCs foci (Fig. 9E). Likewise, we found decoration of microtubules (Fig. 9H and inset picture), but not actin (data not shown), with RPGRIP1s.
Computational and integrated morphometric colocalization analysis of RPGRIP1s to NPCs in 661W cells

To estimate the fraction of NPCs associated at a given point in time with RPGRIP1s, and the fraction of nuclear RPGRIP1s localized at NPCs, we carried out integrated morphometric analysis of $z$-serial focal and deconvolved planes of nuclei with uniform spatial distribution of NPCs (identified with mAb414). The spatial coordinates of NPCs and RPGRIP were then fed into a custom based algorithm for computational analysis of colocalization of RPGRIP1s to NPCs (see Materials and Methods). In addition, we compared the data obtained for RPGRIP1s identified with the antibodies, MCW3 and Ab 38. In contrast with the latter, the former detects a subset of RPGRIP1 isoforms (Fig. 4B) (17).

Figure 9. RPGRIP1s colocalize to NPCs and microtubules at restricted foci in the murine photoreceptor transformed cell line, 661W. (A) 661W cells strongly express the RPGRIP1 isoforms, p150 and p45 (lane 3, 120 µg). In addition, they also express p175 (only observed in bovine retinal tissue, see Figs 5 and 6) and a novel isoform, p62, just barely detected in rat (lane 1) but not in mouse (lane 2) retina-detergent solubilized extracts (120 µg). Western blots were carried out with Ab 38 against RID of RPGRIP1. (B) mAb414 recognizes RanBP2, Nup153 and Nup62 in western blots of 661W cell homogenates. (C–H) Focal planes from serial Z-stack (raw) images immunostained against RanBP2/Nup153/Nup62 (C), RPGRIP1 (D, G), and $\alpha$-tubulin (F) with the mAb414, Ab 38 and anti-$\alpha$-tubulin antibodies, respectively. (E and H) Are overlay images of (C and D) and (F and G), respectively. The inset in (H) is a magnified image of the boxed region in (H) showing the colocalization (arrows) of RPGRIP1s at spaced foci along microtubules. Note RPGRIP1s are localized along the nuclear rim, inside the nuclear compartment and in the perikarya as dispersed puncta and filamentous-like formations (D). In addition, RPGRIP1s are observed occasionally in spatial restricted compartments of the perikarya as coiled-like inclusion bodies (arrowheads, G). The presence of such bodies appears to correlate with the absence of microtubules in that region. RPGRIP1s colocalize to a subpopulation of NPCs foci and often interface with these at the nuclear rim (E, arrowheads).
As shown in Figure 10, the colocalization analysis carried out with Ab 38 (B) and MCW3 (F), and of hundreds of NPCs puncta detected with mAb414 (A, E), were calculated and plotted (centroids X and Y refers to the mass center of the puncta and their coordinates). (C and G) Colocalization coordinates of (A and B), and (E and F), respectively. (D and H) Deconvolved and overlay images of RPGRIP1s and NPCs used for colocalization computation in (C and G), respectively. The nucleus on the left in (D) was employed for the computations shown in (A–C). Green and red colors in (D and H) are for NPCs and RPGRIP1s, and yellow denotes colocalization. Note Ab 38 detected an increased number of RPGRIP1s foci localized to NPCs than that obtained with MCW3 antibody, which recognizes a subset of RPGRIP1 isoforms. MCW3 antibody also detected much fewer RPGRIP1s in the cytosolic compartment, possibly due to epitope masking of RPGRIP1s in this compartment.

**DISCUSSION**

Mutations in the *RPGRIP1* gene in the human lead to Leber congenital amaurosis (LCA) (22,23). LCA is an extremely severe retinal dystrophy typically characterized by an early onset of very severe visual impairment and abnormal or absent electroretinograms (ERGs) often by the age of one year (24–26).
These are often associated with other ocular phenotypes (abnormal pupil reflexes, fine pendular nystagmus, narrowed retinal arterioles, photophobia, eye poking, etc.), prominent oculodigital reflexes, macular colobomas and increased keratoconus incidence (48–54). Complicated cases of LCA have been also reported to be associated with other clinical manifestations such as deafness, seizures, delay in development (including mental retardation) and other systemic abnormalities (25,55–62). Many of these extraocular anomalies appear to be of variable expression in LCA patients and the nosologic relation among these diseases in complicated cases of LCA remains unclear. The extreme severity of the retinal phenotypes described is difficult to explain by just a complete loss of photoreceptors and/or function of these. Thus, it is possible that inner retinal neurons play an important role in the pathogenesis of LCA. To this end, RPGRIP1-expressing amacrine neurons may contribute significantly to maintain retinal function and secure the development of a mature retinal circuitry across species. However, the broader expression of RPGRIP1 in neurons of the INL of bovine retinas (Fig. 1A and F) may implicate a role of RPGRIP1 in other neurons of the INL. In any event, the role of inner retinal neurons in the pathogenesis of LCA seems to be further strengthened by the recent observation of LCA patients with mutations in CRB1. These patients exhibit abnormal thick and coarse lamination of retina, instead of its thinning, as is typically observed in other retinal degenerative diseases, such as those caused by mutations in RPES3 and GUICY2D (63). Altogether, these data and the results presented here support the hypothesis LCA-causing genes affect retinal function and/or development by multiple and independent mechanisms, and underscore the need for further work to determine the degree of contribution of loss of photoreceptor function in the pathogenesis of LCA (63,64).

The murine Rpgrip1 gene has recently been disrupted by imperfect homologous recombination with a targeting construct into the Rpgrip1 locus (65). This led to severe defects in disk morphogenesis. However, it is unclear whether the disruption of Rpgrip1 abolished the expression of all Rpgrip1 isoforms, including those expressed in the inner retina (65). Three significant findings on Rpgrip1 reported by the authors (65) are in apparent contradiction with the results here and previously reported (17,18).

First, the apparent molecular mass of murine Rpgrip1 reported is ~210 kDa, while the high molecular mass of the Rpgrip1 isoform in our studies is consistently ~150 and ~175 kDa in the mouse, rat and human, and bovine retina tissues, respectively. These apparent masses match closely the calculated protein masses encoded by the murine (152 kDa) and predicted human (146.7 kDa) transcripts reported for the largest Rpgrip1 isoform (Fig. 4B). We tend to conclude that the mass discrepancies reported may originate from differences in SDS–PAGE procedures and/or retinal homogenate/extract preparation. Moreover, we have shown multiple antibodies against different and unique domains of Rpgrip1 recognize independently the Rpgrip1-immunoreactive isoform of 175 kDa in the bovine, an apparent mass consistently obtained by high-resolution SDS–PAGE in the Hoefer apparatus (Amersham Pharmacia), and the 175 kDa protein is unequivocally Rpgrip1, as confirmed by Edman sequencing of p175 (Fig. 8A and B). The lower predicted mass of 138.4 kDa of the largest bovine Rpgrip1 isoform compared with that observed in SDS–PAGE, suggests that the bovine may express still another longer and yet unidentified transcript.

Second, the authors reported the presence of a single Rpgrip1 isoform in murine retinas. Instead, we find the expression of at least an additional isoform of ~45 kDa in mouse retinas that is highly conserved and abundant among species (Figs 5A and 6). This and other small mass Rpgrip1 isoforms (e.g. p33) are detected only with an antibody against the highly conserved RID of Rpgrip1s (Figs 5A and 6), but not against any other Rpgrip1 domains (Fig. 8). The existence of multiple Rpgrip1 isoforms is further strengthened by the isolation of multiple alternative spliced Rpgrip1 transcripts from the human and bovine (Fig. 4A and B) (18,20), albeit some Rpgrip1s may also be generated by limited proteolysis. The existence of additional Rpgrip1 isoforms is difficult to evaluate in the absence of a significant range of molecular masses of proteins from the SDS–PAGE shown (65).

Third, antibody-dependent recognition of masked epitope/s in Rpgrip1 may account for the lack of immunostaining observed in inner retinal neurons such as amacrine cells (21). This is supported by the perinuclear and nuclear localization of Rpgrip1 in the nucleus upon transfection of COS cells with constructs comprising the N-terminal and conserved C-terminal domains of Rpgrip1 (65), and the lack of reports of any such localization in retinal cells (21). This is in contrast to antibodies reported herein that localized Rpgrip1 at the outer and inner nuclear rim/NPCs of amacrine neurons, and colocalization of Rpgrip1 to NPCs and/or at their vicinity. These observations were also corroborated with the transformed murine photoreceptor-derived cell line, 661W (Figs 9 and 10).

In light of the above discussion, it is also unclear whether the disruption of the Rpgrip1 locus reported abolishes the expression of all Rpgrip1 isoforms because the targeting of Rpgrip1 left intact the genomic region (after exon 13) encoding ~830 amino acids of the C-terminal end of Rpgrip1 (out of the 1331 residues of the large and full-length Rpgrip1 isoform) (65). In light of the alternative spliced transcripts of Rpgrip1 isolated (18,20), Rpgrip1 isoforms identified and reported here, the murine genomic region after exon 13 still has the potential capacity to code for smaller Rpgrip1 isoforms (Fig. 4A and B). Despite these apparent discrepancies, our results are in agreement with several observations reported by the Li laboratory (21,65). Among these are the localization of Rpgrip1 (and Rprg) to the connecting cilium of murine photoreceptors (17,21,65), the insolubility exhibited by the large Rpgrip1 isoforms (e.g. p175/ p150) (21) and the high propensity of the N-terminal domain of the large bovine Rpgrip1 isoform to polymerize/self-aggregate into urea-resistant precipitates (18,65). This property may lead to the formation of the coiled-like inclusion bodies observed in some 661W cells (Fig. 9G), possibly because of the absence of OS and an axoneme in these cells.

To investigate the pathogenesis of LCA caused by mutations in Rpgrip1, we employed a combination of immunocytochemistry, immunochemical, biochemical and subcellular fractionation approaches to investigate the distribution, expression and biochemical properties of Rpgrip1 isoforms among retinal neurons and subcellular compartments thereof. In this report, we found a subset of amacrine cell located at the...
proximal edge of the INL and some at the ganglion cell layer
express strongly RPGRIP1s in several subcellular compart-
ments (Figs 1 and 2). In particular, RPGRIP1s are preponder-
ant at the nuclear rims and axonal processes of a subset of
amacrine cells. At the nuclear rims, RPGRIP1s often colocalize
with components of the nuclear pore complex (NPC), such as
RanBP2/Nup358, and between NPC foci. RPGRIP1 can also
be observed at the outer and inner faces of such complexes
(Fig. 2). These data support the idea that at least some
RPGRIP1s participate in nuclear-cytoplasmic trafficking pro-
cesses. The observation that not all RPGRIP1-immunoreactive
amacrine cells exhibit colocalization of RPGRIP1s with
RanBP2 and/or NPC foci may reflect interacting events
stochastically captured between RanBP2 and RPGRIP1s at
the time of the fixation of the retinas. However, the localization
of RPGRIP1s at the nuclear rim and with NPCs foci appeared
more preponderant in 661W cells (Figs 9D and 10). RanBP2
has been implicated in mediating rate-limiting steps of nuclear-
cytoplasmatic trafficking in light of the docking and release
reactions of transport of cargoes proposed to occur at RanBP2
(36,38,66–68). Thus, it is expected that components participat-
ing in these steps exhibit longer resident times and higher
affinities upon docking to RanBP2 and only a fraction of
RanBP2 molecules should be associated with RPGRIP1
cargoes in transit at a given time. Such model is also supported
by the communoprecipitation experiments where RanBP2 but
not the topologically distinct, Nup153 and Nup62, associated
with RPGRIP1 (Fig. 3D), and a comparable amount of
RPGRIP1 (p175/p150) coprecipitated with antibodies against
just RanBP2 and RanBP2/Nup153/Nup62 (Fig. 3A). Still, one
cannot exclude the participation of other NPC components not
avidly associated with RanBP2/Nup153/Nup62 to interact
transiently with RPGRIP1s.

The association of the bovine RPGRIP1s, p175/p150 and p95
with RanBP2 (Fig. 3) indicates that the RanBP2 scaffold may
serve as a docking station for these RPGRIP1 isoforms upon
transit to and/or from the nuclear compartment. It is unclear why
the antibody against kinesin-binding domain (KBD) of RanBP2
(39) also coprecipitated an RPGRIP1 isoform of 95 kDa (p95) in
addition to p175, the only isoform coprecipitated with two other
independent antibodies against RanBP2 (Fig. 3). In light of the
flexible conformational states adopted by RanBP2 (36), one
possibility is this antibody recognizes broader conformational
states of RanBP2 generated upon the association/docking of the
two RPGRIP1 isoforms. In addition, in light of the association
of the conventional kinesin heavy chains, KIF5C and KIF5B, with
the KBD of RanBP2, it is tempting to speculate that one or more
kinesin isoforms may mediate the transport of RPGRIP1 to/from
RanBP2 in amacrine cells, where KIF5s are highly expressed
(37). Finally, the observation of partial colocalization of
RPGRIP1s at restricted foci along microtubules is supportive of
a microtubule-dependent transport mechanism of RPGRIP1s
(or some of its isoforms; Fig. 9H and inset figure) and/or
participation of these in the remodeling of the microtubule
organization.

The RPGRIP1 isoform, p175 (and p150), is enriched in the
outer segment of photoreceptors. This isoform is partitioned
mostly to the insoluble fraction of the outer segment compart-
ment of photoreceptors, albeit a relatively small but significant
pool remains soluble in the detergent extracted fraction (Fig. 8D).
This is in contrast to all other isoforms, which are solubilized
with detergents. The extraction of RPGRIP1 with NP-40 appears
to render the p175 isoform to limited proteolysis into p95 as this
isoform was not detected in the homogenate of the starting ROS
material (Fig. 8D, compare lanes 1 and 2). Despite the
preponderant abundance of p175 in the outer segment compart-
ment of photoreceptors (Figs 4, 5 and 6D), this isoform was also
clearly detected in highly purified nuclei (Figs 7D and 8E).
Interestingly, p175 seems to be resistant to detergent and urea
extraction of nuclei (Fig. 8E), but these treatments lead to a
dramatic increase of p33. This supports the p33 isoform being
produced by limited proteolysis. The solubility profiles of the
RPGRIP1 isoforms described, susceptibility of RPGRIP1s to
limited proteolysis upon detergent extraction, differential
association of these with RanBP2 as well as their differential
localization among subcellular compartments and retinal
neurons, indicate RPGRIP1 isoforms and/or proteolytic products
thereof are likely to play different roles in photoreceptors and
amacrine cells. The predominant sequestration of p175 in the
cytoskeleton (and outer segment and possibly, connecting
cilium) may reflect a role of this isoform in remodeling the
cytoskeleton of the ROS. In contrast, other role(s) may be
subsumed by other smaller RPGRIP1 isoforms derived from
alternative splicing and/or limited proteolytic byproducts of
p175/p150. These may be released from the cytoskeletal pool
and impart signaling properties and new diversity to RPGRIP1
function(s) in a subset of amacrine cells, and possibly,
photoreceptors. Such a scenario would be reminiscent of that
proposed for the regulated intramembrane proteolysis (RIP) of
SREBP-1, Notch-1, APP and ErbB-4, where limited proteolysis
generates a diverse signaling repertoire of byproducts in the
cell (69). Observations here reported and genetic evidence reported
by others indicate the large RPGRIP1 isoform (p175/p150) plays
a key role in LCA pathogenesis and retinal/photoreceptor
function. First, homozygous frame-shift mutations in exons 4
and 9 leading to premature stop codons (Fig. 4A) of the human
RPGRIP1 affect presumably only the large RPGRIP1 isoforms
(p175/p150) (22,23). Second, the localization of p175 in the
outer segment of photoreceptors and a subset of inner retinal
(amacrine) neurons support a key role of this isoform in these
neurons. Third, selective disruption of the Rpgr1 gene severely compromise photoreceptor function and structural integrity of its OS (65). Fourth, the large RPGRIP1 isoform is expressed
only in the retina, while the other isoforms are expressed in other
tissues (18).

Albeit the focus of this report is on RPGRIP1, the role of
RPGR in RPGRIP1 function should be noted. RPGR may be
essential to RPGRIP1 in the outer segments of photoreceptors,
where they colocalize (17). RPGR function in inner retinal
neurons such as amacrine cells, where RPGR is also expressed
(17), may be mostly redundant in light of the RP phenotype
exhibited by XIRP3 patients. However, a report of disturbances
in integration of rod responses in XIRP3 carriers exists and
rod-driven depolarizing amacrine cells such as AII and A17
subtypes were even postulated to mediate such responses (70).
In any event, the large number of mutations identified in the
XIRP3 locus in the human and clinical characterization of such
patients support RPGR function being mainly required for
photoreceptor function and survival (10). In contrast, RPGRIP1
may subserv additional functions in a subset of amacrine cells

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as supported by the data reported herein. RPGRIP1’s functions in these neurons possibly reflect the severity of phenotypes observed in humans with mutations in RPGRIP1 and expressing LCA instead of RP (22,23).

MATERIALS AND METHODS

Tissue sources, primary antibodies and reagents

Fresh bovine (Bos taurus) eyes with a post-mortem time of less than 30 min were obtained from a local slaughterhouse. Human eyes with a post-mortem time of 6–12 h were provided by the Eye Bank at the Eye Institute of the Medical College of Wisconsin. Dog retinas were kindly provided by Gustavo Aguirre (Cornell University, Ithaca, NY, USA). Mice retinas were from 6-month-old C57Bl/6. Mice eyes and rat retinas were Enucleation and processing of bovine retinas were performed between 11 a.m. and 1 p.m. All tissue manipulation procedures complied with institutional and federal guidelines. The affinity-purified antibodies, MCW3 and MCW4, Ab 22 and Ab 23, and Ab 38 and Ab 39, respectively, against the C2 domain of RPGRIP1 N- (SMC) and RID C-terminal domains of RPGRIP1 have been described previously (17,18). The monoclonal antibodies against RanBP2/Nup153/Nup62 (mAb414), and nuclear pore-O-linked glycoproteins, rhodopsin and myosin VIIa, were from Convenance (Richmond, CA, USA) and Affinity Bioreagents (Golden, CO, USA), respectively. The affinity-purified polyclonal antibodies, ZnF2090 and JX41, and Ab 21069, against specific domains of RanBP2 and bovine rhodopsin, respectively, have been described elsewhere (17,38,39). Antibodies against importin-β, Nur77, LamA/C and β-tubulin were purchased from Transduction Laboratories (Beverly, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technologies (Beverly, MA, USA) and Sigma (St Louis, MO, USA), respectively. The filamentous actin stain, Alexa594-phalloidin, was from Molecular Probes (Eugene, OR, USA). Prestained and unstained SDS–PAGE markers were from New England Biolabs (Beverly, MA, USA).

Immunohistochemistry of retina sections and computation of colocalization

Retina dissections, radial cryosections of these and immunohistochemistry procedures were carried out exactly as described elsewhere (17,37) with the exception that all retinal sections were permeabilized, incubated and washed with 0.1% Triton X-100. All primary antibodies were used at the concentrations given in the figure legends. Goat anti-rabbit and -mouse antibodies conjugated with fluorescent dyes (Molecular Probes), Alexa 488 and Alexa594, were used at the concentration of 2.5 μg/ml. Visualization of retinal sections and localization of proteins were carried out by wide-field epifluorescence microscopy on upright E600 and inverted TE2000 Nikon research microscopes. Procedures of image capturing on E600 microscope were performed as previously described (17,37). Protein colocalization analyses were carried out on the TE2000 Nikon microscope equipped with appropriate excitation and emission filter wheels, cube filters, 100 W mercury light source, Nomarski/DIC and Plan Apochromat optics (100×, 60× and 40× oil objectives with NA of 1.4, 40× LWD and 20× LWD objectives and encoded motorized Z-Stage. Images were obtained using a CCD camera (CoolSNAP HQ: Ropers Scientific) and under identical acquisition parameters, and were analyzed using Metamorph Software version 6.1 (Universal Imaging). Whenever applicable, serial optical Z-stacks (20–40 focal planes at 100 nm intervals) were captured and computationally processed by two- and/or three-dimensional blind deconvolution methods in Metamorph Software version 6.1 (Universal Imaging). Merged images were carried out with the same software. Overall arrangements and cropping of images were performed by importing them to Adobe Photoshop version 5.5 (Adobe, Mountain View, CA, USA).

Computation of colocalization of two given proteins was performed in Metamorph Software version 6.1 (Universal Imaging) by thresholding the deconvolved images to subtract the average background fluorescence and obtaining integrated morphometric analysis of two sets of puncta with central x-y coordinates and equivalent radii (centroids X and Y). For colocalization of NPCs with RPGRIP1s, the perikarya was excluded from analysis. Then, the two sets of data obtained were fed into a custom-written algorithm (Visual Basic). The average equivalent radius of two sets of puncta was calculated and used to filter out any gross background puncta. Puncta with radius smaller than or equal to the average equivalent were kept. All the distances between any two sets of puncta derived from two images were calculated. If the distance between two puncta coordinates was smaller than or equal to the sum of their radii, those two puncta were considered colocalized. The percentage of colocalized puncta was then calculated and colocalized puncta were plotted.

Preparation of retinal homogenates and western blot analysis

Homogenates of bovine, human, dog and rat retinas and of mice eyes were prepared as previously described (18). Briefly, the tissue was homogenized in 3 × SDS-sample buffer (5% w/v SDS, 0.15 M Tris–HCl, pH 6.7, and 30% glycerol) by several passes through increasingly narrower needles (18G1, 22G1 and 25G5/8, respectively) followed by 1 : 3 dilution in RIPA buffer [25 mM Tris, pH 8.2, 50 mM NaCl, 0.5% Nonied P-40 (NP-40), 0.5% deoxycholate and 0.1% SDS] containing 10 mM iodoacetamide and Complete protein inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein concentration was measured by the BCA method using BSA as the standard. All protein samples were boiled unless otherwise stated and then resolved on SDS–PAGE, and western blots were carried out as described elsewhere (71). Dilutions of antibodies used are specified in figure legends. Blots were developed with a SuperSignal chemiluminescence substrate (Pierce, Rockford, IL, USA).

Subcellular fractionation of the retina and nuclei isolation

Ten bovine retinas (∼0.5 g each) were homogenized (1 : 6) in 0.25 M sucrose in 50 mM Tris, pH 7.5 containing 25 mM KCl, 5 mM MgCl₂ (TKM) and 0.2 mM CaCl₂ in a Dounce homogenizer with 10 strokes using the loose pestle (A).
The homogenate was first filtered through four layers of cheese cloth and centrifuged at 1000g for 10 min. The pellet (crude nuclear pellet) was further purified by resuspending it in 9 ml 0.25 M sucrose in TKM, 0.2 mM CaCl₂, pH 7.5, mixing it with 2.3 M sucrose solution in TKM (1:2, yields a 1.6 M sucrose homogenate) overlaying this on 2.00 M sucrose solution in TKM and spinning this sucrose barrier system at 100 000 g for 1 h using an SW28 rotor. The purified nuclei were recovered as a pellet (Nuc 1). The other retinal organelles floated at the 1.6–1.75 interface (Nuc 2) and crude rod outer segments (ROS) floated at the top of the gradient. The supernatant obtained in the first centrifugation step was further centrifuged at 17 000g for 15 min. The resulting pellet was the crude mitochondrial fraction (Mit) and the supernatant was the post-mitochondrial fraction (Postmit). The crude ROS were then purified by a discontinuous sucrose gradient as previously described (72). Purified ROS were retained at the 0.84/1.0 M sucrose interphase. This band floated in sucrose-free TKM (1 : 2, yields a 1.6 M sucrose barrier system were diluted with sucrose-free TKM containing 0.2 mM CaCl₂ (1 : 2) and further centrifuged at 30 000 g for 4 min. The ROS-free retinas were sedimented at 3 000g for 30 min and the supernatants containing ROS were diluted 1:2 with sucrose-free buffer, followed by 30 min of centrifugation at 31 000g. The crude ROS were then purified by a discontinuous sucrose gradient as previously described (72). Purified ROS were retained at the 0.84/1.0 M sucrose interphase. This band was collected and diluted with 70 mM sodium phosphate buffer (pH 7.2) and centrifuged at 31 000g for 30 min. All the resulting pellets were resuspended in NP-40 or RIPA buffer, aliquoted and stored at −70°C until further use.

Rod outer segment isolation

Retinas were dissected from the bovine eyes and all subsequent isolation procedures were conducted at 2–4°C. Rod outer segments (ROS) were detached by shaking the retinas twice in a 40% sucrose solution containing 1 mM MgCl₂, 1 mM DTT and complete protein inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) in 70 mM sodium phosphate buffer (pH 7.2). The ROS-free retinas were sedimented at 3 000g for 4 min and the supernatants containing ROS were diluted 1:2 with sucrose-free buffer, followed by 30 min of centrifugation at 31 000g. The crude ROS were then purified by a discontinuous sucrose gradient as previously described (72). Purified ROS were retained at the 0.84/1.0 M sucrose interphase. This band was collected and diluted with 70 mM sodium phosphate buffer (pH 7.2) and centrifuged at 31 000g for 30 min. All the resulting pellets were purified ROS. Electron micrographs from purified ROS presented intact outer segments and no other membrane material was observed (data not shown).

Ultrastructural characterization of nuclear fractions

Purified nuclei were fixed in a mixture of 2% glutaraldehyde/1% paraformaldehyde in 0.1 M PBS, pH 7.4 for 30 min at room temperature, centrifuged twice at 1000 g for 5 min, postfixed in 2% glutaraldehyde for 1 h, rinsed three times and postfixed in 1% OsO₄ for 2 h. Then, samples were dehydrated using increasing concentrations of alcohol: 50% (twice), 70%, 80, 90 and 100% (twice), followed by propylene oxide treatment for 1 h (twice). The samples were then embedded in a mixture of propylene oxide/EPOX (1:1 for 24 h, 1:3 for 24 h, 100% EPOX for 24 h at room temperature), and polymerized in 100% EPOX for 24 h at 60°C. Blocks were cut across the pellet to 50 nm thin sections, contrasted by uranyl acetate and lead citrate. Micrographs were taken under a Hitachi electron microscope.

Protein sequencing analysis of p175 RPGRIP1 isoform

P175 was immunopurified with Ab 38 from bovine retinal extracts, resolved on SDS–PAGE, blotted onto PVDF membranes, stained with 0.1% Coomassie blue in 50% methanol and 1% acetic acid and washed several times in destaining solution (50% methanol, 1% acetic acid) followed by several washes with Millipore H₂O as reported elsewhere (73). The 175 kDa band was cut and subjected to Edman degradation at the Cancer Center Mass Spectrometry Resource Laboratory of Yale University.

661W cell culture

The 661W cells were grown in DMEM supplemented with 10% (vol/vol) fetal calf serum, 2 mM l-glutamine and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. Cells were grown to ~40–60% confluence and processed for immunocytochemistry. Homogenates of 661W cells for western analysis were prepared from confluent grown cells by washing attached cells with PBS followed by cell lysis in 3× SDS-sample buffer and 1:3 dilution in RIPA buffer as previously described.

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NOTE ADDED IN PROOF

Following submission of this manuscript, a report by Hong et al. revealed different apparent molecular masses among the large Rpgrp1 isoform of mouse and rat, and localization of RPGR solely to the connecting cilia of bovine photoreceptors; these data are inconsistent with ours [Hong, D.H., Pawlyk, B., Sokolov, M., Strissel, K.J., Yang, J., Tulloch, B., Wright, A.F., Arshavsky, V.Y. and Li, T. (2003) RPGR isoforms in photoreceptor connecting cilia and the transitional zone of motile cilia. Invest. Ophthalmol. Vis. Sci., 44, 2413–2421].

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