Human retinopathy-associated ciliary protein retinitis pigmentosa GTPase regulator mediates cilia-dependent vertebrate development

Amiya K. Ghosh, Carlos A. Murga-Zamalloa, Lansze Chan, Peter F. Hitchcock, Anand Swaroop and Hemant Khanna

1Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, MI 48105, USA and 2Neurobiology-Neurodegeneration and Repair laboratory (N-NRL), National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA

Received May 28, 2009; Revised September 30, 2009; Accepted October 6, 2009

Dysfunction of primary cilia is associated with tissue-specific or syndromic disorders. RPGR is a ciliary protein, mutations in which can lead to retinitis pigmentosa (RP), cone-rod degeneration, respiratory infections and hearing disorders. Though RPGR is implicated in ciliary transport, the pathogenicity of RPGR mutations and the mechanism of underlying phenotypic heterogeneity are still unclear. Here we have utilized genetic rescue studies in zebrafish to elucidate the effect of human disease-associated mutations on its function. We show that \( rpg \) is expressed predominantly in the retina, brain and gut of zebrafish. In the retina, RPGR primarily localizes to the sensory cilium of photoreceptors. Antisense morpholino (MO)-mediated knockdown of \( rpg \) function in zebrafish results in reduced length of Kupffer’s vesicle (KV) cilia and is associated with ciliary anomalies including shortened body-axis, kinked tail, hydrocephaly and edema but does not affect retinal development. These phenotypes can be rescued by wild-type (WT) human RPGR. Several of the RPGR mutants can also reverse the MO-induced phenotype, suggesting their potential hypomorphic function. Notably, selected RPGR mutations observed in XLRP (T99N, E589X) or syndromic RP (T124fs, K190fs and L280fs) do not completely rescue the \( rpg \)-MO phenotype, indicating a more deleterious effect of the mutation on the function of RPGR. We propose that RPGR is involved in cilia-dependent cascades during development in zebrafish. Our studies provide evidence for a heterogenic effect of the disease-causing mutations on the function of RPGR.

INTRODUCTION

Primary cilia are microtubule-based extensions of the plasma membrane generated by Intraflagellar transport (IFT) of precursor proteins in almost all post-mitotic cells (1). Once considered vestigial, the primary cilia are now recognized to perform diverse and essential cellular functions, including cell cycle, protein trafficking, embryonic axis patterning and photoreception (2–6). Defects in ciliary function are associated with syndromic disorders including Senior-Loken Syndrome, Bardet–Biedl Syndrome and Joubert Syndrome (7). Many of these disorders include retinal degeneration, RP and LCA (Leber congenital amaurosis) due to perturbed photoreceptor ciliary transport (7,8).

Mutations in retinitis pigmentosa GTPase regulator (RPGR) are a major cause of photoreceptor degenerative diseases, including X-linked and simplex forms of RP, cone-rod degeneration and atrophic macular degeneration (9–15). Some RPGR patients exhibit hearing dysfunction, respiratory infections and primary cilia dyskinesia (16–21). Multiple alternatively spliced isoforms of RPGR are widely expressed, at both RNA and protein levels (22–24). Two major isoforms of RPGR are detected in the retina: RPGR\(^{1–19}\) (amino acids 815; exons 1–19) and RPGR\(^{1–ORF15}\) (amino acid residues...
1152; exons 1–15 and part of intron 15) (10,13). Both iso-
forms share exons 1–15 (residues 1–635) and encompass a
common N-terminal RCC1-like domain (RLD; residues 1–
446) (10,13). Although RCC1 is a guanine nucleotide
exchange factor for Ran GTPases (25), no such activity has
yet been reported for RPGR. Mutations in exons 1–15 of
RPGR account for almost 20% of XLRP with no
disease-associated variations reported in exons 16–19 (12).
The purine-rich RPGR exon ORF15 encodes a Glutamic
Acid and Glycine (Glu–Gly) rich C-terminal domain;
mutations in this exon account for additional 50–60% of
XLRP (12,13).

The RPGR protein isoforms are prominently localized to the
sensory cilia of photoreceptors (transition zone and basal
bodies), human and monkey cochlea and to the nucleus,
cilia and centrosomes of cultured cells (16,23,26–28).
RPGR is shown to interact (directly or indirectly) with
several ciliary proteins, including RPGR-interacting protein
1 (RPGRIP1). RPGRIP1-like (RPGRIP1L/NPHP8) (29) and
CEP290/NPHP6 (2,3,23,27,30–32). Given that CEP290 and
RPGRIP1L regulate ciliogenesis and cilia-dependent signaling
(33,34), we hypothesize that RPGR is essential for primary
cilia genesis or function. Studies using mouse and canine
models of RPGR mutations demonstrate phenotypic hetero-
genity and altered ciliary transport (27,35,36). Within this
context, the mechanism(s) by which RPGR mutations cause
diverse phenotypes in humans have not been defined at the
biochemical or cellular level.

We and others have shown that knockdown of expression of
ciliary proteins in zebrafish (Danio rerio) results in measur-
able structural and functional phenotypes, some of which are
due to perturbed planar cell polarity (PCP) or vesicular traf-
ficking (5,29,37–39). Ability of human mRNA to genetically
rescue the ciliary phenotype serves as an excellent platform to
assess the functional consequence of the human disease-
causing mutations by comparing the efficacy of rescue to the
wild-type counterpart. We have utilized the zebrafish system
to examine the function of RPGR and pathogenicity of the
retinopathy-associated mutations on its function. We show
that RPGR is essential for cilia-dependent developmental
pathways in zebrafish. Moreover, some human disease-causing
RPGR mutations, which result in syndromic or isolated RP in
patients, exhibit a loss-of-function effect while others seem to
be hypomorphic. These studies provide novel insights into the
function of RPGR in cilia-dependent pathways and reveal
critical information about variable pathogenic consequences
of the disease-causing mutations in RPGR on its function.

RESULTS

RPGR is expressed in zebrafish during development

Using BLAST analysis, we first identified an rpgr transcript
(Accession number XM_680872.3), which bears 57% identity
and 72% similarity to human RPGR<sub>ORF15</sub>. This rpgr transcript
consists of 14 exons with terminal exon encoding for
purine-rich repeats (Fig. 1A). Reverse transcriptase-
polymerase chain reaction (RT–PCR) analysis of RNA from
zebrafish embryos revealed an expected rpgr cDNA product
of 4.2 kb (Fig. 1A). Whole-mount immunofluorescence
analysis using a previously reported rabbit polyclonal antibody
(GR-P1) against human RPGR-RLD (27,40) revealed that
RPGR is expressed predominantly in the eye, head and gut of
4 days post-fertilization (dpf) zebrafish embryos (data not
shown). Further analysis of zebrafish embryos at 5 dpf
revealed RPGR expression predominantly in the brain, optic
chiasma and gastric epithelial layers (Fig. 1B and C). In
the retina at 5 dpf or adult zebrafish, RPGR localizes predomi-
nantly in the inner segment/sensory cilium region of photo-
ceptors, where it co-localizes with acetylated α-tubulin (Fig. 1D;
Supplementary Material, Fig. S1). The staining of
RPGR in different tissues of zebrafish embryos can be
blocked by pre-incubating with the specific antigen but not
by a non-specific antigen (data not shown).

Knockdown of RPGR function causes ciliary
defects in zebrafish

We then proceeded to evaluate the effect of knockdown of rpgr
expression in zebrafish. Injection of translation-blocking mor-
pholino (MO) against rpgr into wild-type (WT) embryos
revealed a dose-dependent defect reminiscent of a ciliary pheno-
type (Fig. 2A). A 3 ng dose of the specific MO against rpgr
resulted in ~60% morphants after 4 dpf, as opposed to ~10%
with a mismatch morpholino (Mm) at the same dose (Fig. 2B).
Embryonic lethality was observed at doses greater than 3 ng of
rpgr-MO. Immunoblot and immunofluorescence analyses
using the GR-P1 antibody confirmed knockdown of RPGR
protein expression in 4–5 dpf morphants (Fig. 2C; Supplemen-
tary Material, Fig. S2). To directly correlate a ciliary defect due
to knockdown of rpgr function in zebrafish, we analyzed the
cilia of Kupffer’s vesicle (KV), a ciliated organ involved in left-
right axis patterning during zebrafish development (5,41–43).
Staining with anti-acetylated α-tubulin revealed that although
the number of cilia is not significantly altered, rpgr-MO
embryos show significant decrease (~50%) in the length of
the KV cilia (Fig. 2D and E). These data indicate that RPGR
is involved in the maintenance of the cilium and not ciliogenesis.

Ciliary dysfunction in zebrafish is frequently associated with
convergent-extension (CE) defects, including body axis exten-
sion anomalies (5,29,38,44,45). We therefore analyzed
rpgr-morphants for such anomalies. As shown in Figure 3A,
rpgr-MO results in kinked tails in 60% of morphants. We also
observed hydrocephaly in ~30% and edema in ~50% of the
morphants, a phenotype also associated with ciliary dysfunction
(46). We also assessed retinal morphology and trafficking of
opsins in the 4–5 dpf abnormal embryos that exhibited other
ciliary anomalies. Notably, the architecture of the retina and
photoreceptors as well as transport of rhodopsin to outer seg-
ments seems unaltered in the defective embryos as compared to
WT embryos (Supplementary Material, Fig. S2).

rpgr-MO phenotype can be rescued by human RPGR

We next investigated whether human WT RPGR can rescue
rpgr-dependent cilia phenotype. We show that the effect of
knockdown of rpgr can be rescued in a dose-dependent
manner using mRNA encoding human RPGR<sub>1–19</sub>
(NM_000328.2), RPGR<sub>1–ORF15</sub> (NM_001034853.1) and
RPGR<sub>1–15</sub> (Fig. 3B). While 3 ng of rpgr-MO yielded 60%
morphants, co-injection of 500 pg of human RPGR mRNA resulted in <10% morphants. These results suggest that the observed MO phenotype is specifically due to the knockdown of rpgr. Co-injection of human WT RPGR mRNA can rescue almost all of the phenotypes detected in rpgr-MO, including length of the KV cilia, as depicted in Figure 3B–D.

**RP-associated mutations in RPGR exhibit hypomorphic and loss-of-function effect**

We then utilized rpgr-MO as a platform to assess the pathogenic potential of selected RPGR mutations found in patients. We first examined selected missense mutations reported in the RLD of RPGR (Fig. 4A). Co-injection of human RPGR mRNA encoding mutants G60V (47), G165V or G173R (12) could rescue the rpgr-knockdown phenotype comparable to that by wt RPGR-encoding mRNA (~15% morphants), whereas RPGR-T99N (48) mutant did not rescue the phenotype (~47% defective embryos) (Fig. 4B and D). Specifically, the T99N mutant can partially rescue the kinked tail phenotype but not edema and hydrocephaly (Fig. 4D; Supplementary Material, Fig. S3).

Next, we assessed the effect of nonsense mutations in exons 1–15 or in exon ORF15 on the function of RPGR.
A termination in exon 15 of RPGR (E589X; c.1765G > T) (12) did not seem to rescue any of the rpgr-MO phenotypes tested (~65% defective embryos) (Fig. 4C and D; Supplementary Material, Fig. S3). Co-injection of mRNA encoding wt RPGR1-ORF15, RPGR1-ORF15 E853X [c.2557G > T] (49) or RPGR1-ORF15 E1071X [c.3211G > T] (50) with rpgr-MO resulted in a comparable rescue (~15% morphants) (Fig. 4C).

RPGR mutations associated with syndromic phenotype cannot rescue the rpgr-MO phenotype

We also assessed three truncation mutations in the RLD exhibiting syndromic phenotypes: T124 [G > T at splice site of intron 5] (14), K190 [631_IVS6 + 9del] (13) and L280 [845–846delTG] (15). These mutations are associated with sino-respiratory infections, pseudo-Usher syndrome and sperm defects in RPGR patients. None of these mutants
could rescue of the rpgr-MO phenotype (40–60% morphants); hence, these mutations appear to be detrimental to RPGR function (Fig. 4C and D). Notably, mutants K190X and L280X cannot rescue the kinked tail, edema and hydrocephaly due to knockdown of rpgr function, whereas T124X exhibited relatively less number of defective embryos. The expression levels of these mutant RPGR proteins in vitro were not altered, indicating that the mutations do not affect the stability of the RPGR protein (Supplementary Material, Fig. S3).

DISCUSSION

There is considerable clinical heterogeneity associated with retinal degenerative diseases due to mutations in ciliary proteins. Investigations on the impact of disease-causing mutations on the function of the causative protein have been difficult due to the lack of a platform to analyze protein function. In this study, we have utilized zebrafish development as a paradigm to elucidate the pathogenic potential of selected disease-associated mutations in RPGR. We show that RPGR mutations exhibit variable effects on its ability to rescue the rpgr-knockdown phenotype in zebrafish. All patients carrying RPGR mutations exhibit photoreceptor degeneration; however, we did not detect any defects in photoreceptor development or opsin trafficking in rpgr-morphants by 4–5 dpf (Supplementary Material, Fig. S2). These results likely suggest that RPGR is not essential for retinal differentiation and development. Notably, RPGR patients and animal models exhibit normal photoreceptor development but undergo relatively late-onset photoreceptor degeneration and blindness (15,35,36). Hence, we cannot rule out an effect on photoreceptor survival in adult zebrafish, as also proposed with bbs genes (5). A lack of retinal phenotype in
RPGR knockdown zebrafish embryos is not unexpected. Because of the duplication of the zebrafish genome (51), a functional redundancy of RPGR is highly likely. Tissue- and cell-type specific alternative isoforms of RPGR have also been reported in mice, bovine and human tissues. Hence, it is possible that the specific isoform of RPGR, investigated in the present study, may not play a role in retinal development. While this study was going on, we identified another potential RPGR isoform by *in silico* analysis of the updated genome of zebrafish. Further investigations are necessary to delineate the function of this isoform in retinal development in zebrafish.

Our studies provide *in vivo* evidence for the functional significance of the N-terminal domain of RPGR (encoded by exons 1–15) in ciliary functions (23). The T99 residue is highly conserved during evolution as well as in the RCC1 protein (10); hence, the T99N mutation may result in altered conformation, a detrimental effect on an as yet uncharacterized activity of RPGR, ciliary localization and/or interaction with other proteins. Truncation mutations in RPGR-RLD that are associated with a syndromic phenotype in patients exhibit an expected detrimental effect on protein function whereas the G173R mutation (associated with hearing loss, sinusitis and recurrent respiratory infections) (17) seems to retain partial function in zebrafish development.

Mutations in exon ORF15 exhibit a hypomorphic effect in our assays. These data are consistent with previous observations that ORF15-variants in patients are associated with a relatively mild phenotype (12,50,52). Moreover, the RPGR<sup>1-ORF15</sup> variants encode the RPGR-RLD as part of exons 1–15, which are sufficient to rescue the *rpgr*-MO phenotype. Intriguingly, animal models of exon ORF15 mutations
exhibit discordant phenotype (slow or rapid photoreceptor degeneration and a dominant gain of function effect) based upon the length of the Glu–Gly repeats encoded by exon ORF15 (36,53). We predict that relative dosage of the truncated ORF15-mutant proteins may alter the functional conformation of the RPGR protein, resulting in a mild versus severe phenotype in the cognate model system. Further studies are necessary to piece-together the complex association of mutations in exon ORF15 of RPGR with its function.

PCP proteins, which localize to cilia, control CE movements during axis elongation in vertebrates (44,54). Our data suggest that RPGR is essential for vertebrate embryonic development and complete loss of RPGR function during development likely results in lethality. Support of this hypothesis also comes from previous observations that RPGR is expressed in mouse embryonic stem cells (unpublished data), and that attempts to generate a complete loss of function mouse mutant of Rpgr have so far been unsuccessful. Moreover, the reported Rpgr-ko mouse (35) is not complete null because expression of alternative RPGR isoforms can be detected in the photoreceptors (23). However, the precise function of RPGR during vertebrate development remains to be established.

Why do RPGR mutations not exhibit lethality in humans? We suggest that a majority of RPGR mutations detected in RP patients are hypomorphs with reduced function. This functional retention seems to be sufficient to rescue the developmental phenotype. Only selected mutations in RPGR may severely compromise the protein function. Given high protein trafficking demands due to periodic outer segment disc shedding and renewal (55–57), even hypomorphic RPGR mutations have deleterious impact on the integrity of ciliary protein complexes and intracellular transport in photoreceptors. We predict that multiple isoforms of RPGR (generated by alternate splicing or with alternate promoter) may complement the detrimental effects of such mutations in extra-retinal tissues in higher vertebrates during development.

Although previous studies have reported a predominantly retinal phenotype associated with RPGR-T99N and RPGR-E589X mutations, we propose that detailed clinical analysis of patients predicted to have deleterious mutations based on the zebrafish assays reported here should be performed to analyze potential heterogenic phenotype(s), such as olfactory dysfunction, which can assist in early diagnosis of the disease (58). We and others have shown that hypomorphic mutations in the ciliary protein CEP290 are associated with predominantly LCA and olfactory defects (58,59) whereas predicted null mutations cause Joubert Syndrome, Bardet–Biedl Syndrome and Meckel–Gruber Syndrome (38,60–63). Although olfactory dysfunction was not significantly observed in the RPGR patients tested (58), additional studies using patients with other mutations should be performed for such analyses.

Taken together, our work analyzes the effect of RPGR mutations on its function using a zebrafish model system. This system offers a platform to understand the behavior of mutant RPGR in vivo so that future therapeutic strategies can be appropriately designed to target a complete or partial loss of function of the mutant RPGR protein in patients with ciliary disorders.

MATERIALS AND METHODS

RT–PCR analysis, immunoblotting and immunohistochemistry

Total RNA was extracted from the 3 dpf embryos using Qiagen RNA extraction kit. RNA was reverse-transcribed to cDNA using oligo-dT. Two milliliters of cDNA was amplified for the full-length RPGR with the following primers:

F1: 5'-GCAGAGATGG CTGGAGAAC-3'
R1: 5'-GCTTTTGCTTATCATCATTTGCT-3'
R2: 5'-AAAACATCATCCAAAACCTG-3'.

Immunoblotting was performed using embryo lysates generated after treatment in radioimmunoprecipitation (RIPA) buffer. The cell lysates were resolved on SDS–PAGE and immunoblotting using appropriate antibodies. For retinal immunohistochemistry, eye sections from zebrafish embryos (4 or 5 dpf) were stained with appropriate antibodies. Fluorescent images were taken using confocal microscope (Leica).

Morpholino and mRNA injections

A translation blocking rpgr-MO (MO; 5'-CTTCTGTTTTCTCAGCCATCTCTGC-3') and its 5 base mismatch morpholino (Mm; 5'-CTTGTGTTTGTcAGcATCTgTGC-3') were purchased from Gene Tools Inc., diluted in Danieau’s solution (5 mM HEPES pH 7.6, 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM calcium nitrate) and injected into WT zebrafish embryos at 1–8 blastomere stage at different concentrations. Different doses of morpholino were injected along with 0.1% Phenol Red into fertilized eggs from AB wild-type breeders at two-cell stage using Picospritzer II microinjector (General Valve Corporation). Embryos were analyzed at 2 dpf for hydrocephaly and 3 dpf for the curved tails and edema phenotypes. For rescue experiments, the RNA was transcribed from human RPGR1–19, RPGR1–ORF15 and RPGR1–19 plasmids with appropriate primers using mMESSAGE mMACHINE kit (Ambion, Austin, TX) in combination with T7 RNA polymerase. RPGR constructs encoding mutant RPGR protein were used as template to generate appropriate mRNA encoding human mutations.

Staining of KV cilia in zebrafish embryos

Embryos at 10–12 somite stage were fixed in 4% paraformaldehyde and processed for staining as described (5). Embryos were incubated overnight with anti-acetylated α-tubulin (Sigma; 1:500 dilution) and GR-P1 (1:250 dilution) antibodies, washed with PBST for 2 h and incubated with anti-mouse Alexa Flour 594 and anti-rabbit Alexa Flour 488 (Molecular Probes). After further washing and removal of the yolk, the embryos were mounted with dorsal side facing upward with the mounting media. Z-stack images of KV cilia were taken using confocal microscope (Leica).

Phenotypic analysis of zebrafish embryos

Embryos were assessed for tail extension anomalies, hydrocephaly and edema essentially as described (29).
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

The authors thank Dr Nicholas Katsanis, Dr Edwin Oh, Dr Weibin Zhou and Dr Friedhelm Hildebrandt for valuable discussions on the phenotypic analysis of zebrafish; Dr Mithu De, Stephen Atkins, Laura K. Atkins and David Nguyen for critical discussion and technical assistance. P.F.H. is a Senior Scientific Investigator of the Research to Prevent Blindness, Inc.

Conflict of Interest statement. None declared.

FUNDING

This work is supported by NEI intramural funds and grants from the National Institutes of Health (grant numbers RO1-EY007961 and R01-EY07060); Midwest Eye Banks and Transplantation Center and Foundation Fighting Blindness. This work also utilized the services provided by the Morphylogy Core of the Michigan Diabetes Research and Training Center (NIH5P60 DK20572) and of the Vision Core Facilities (EY07003).

REFERENCES


