Biochemical Characterization and Subcellular Localization of the Mouse Retinitis Pigmentosa GTPase Regulator (mRpgr)*

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The retinitis pigmentosa GTPase regulator (RPGR) gene encodes a protein homologous to the RCC1 guanine nucleotide exchange factor and is mutated in 20% of patients with X-linked retinitis pigmentosa. We have characterized the full-length and variant cDNAs corresponding to the mouse homolog of the RPGR gene (mRpgr). Comparison with the human cDNA revealed sequence identity primarily in the region of RCC1 homology repeats. As in humans, the mRpgr gene maps within 50 kilobases from the 5'-end of the Otc gene. The mRpgr transcripts are detected as early as E7 during embryonic development and are expressed widely in the adult mice. Variant mRpgr isoforms are generated by alternative splicing and by utilizing two in-frame initiation codons. The products of mRpgr cDNAs migrate aberrantly in SDS-polyacrylamide gels because of a charged domain. In transfected COS cells, the mRpgr protein is isoprenylated and is localized in the Golgi complex. This subcellular distribution is not observed after treatments with brefeldin A or mevastatin and when the conserved isoprenylation sequence (CTIL) at the carboxyl terminus is deleted or mutagenized. These studies suggest a role for the mRpgr protein in Golgi transport and form the basis for investigating the mechanism of photoreceptor degeneration in X-linked retinitis pigmentosa.

Retinitis pigmentosa (RP)† refers to a group of retinal degeneration disorders characterized by night blindness, progressive loss of peripheral vision, and a characteristic pigmentary retinopathy (1). In addition to wide variations in clinical phenotype, RP exhibits extensive genetic heterogeneity; more than 15 distinct genetic loci have been localized to human chromosomes, and mutations in a large number of genes have been associated with inherited retinal degeneration (2; also see RetNet web site www.sph.uth.tmc.edu/www/utsph/RetNet/ disease.htm). Patients and carriers with X-linked forms of RP (XLRP) demonstrate severe retinitis pigmentosa and account for 7–30% of the RP population (3, 4). Two major XLRP loci, RP2 and RP3, have been mapped to Xp11.3-p11.23 and Xp21.1, respectively; of these, RP3 is the more common form accounting for 70% of XLRP (4, 5). Sequencing of the genomic DNA spanning small deletions in RP3 patients revealed a novel gene, RPGR (retinitis pigmentosa GTPase regulator), which is shown to be mutated in 20% of XLRP families (6–8). As yet unidentified exon(s) in the RPGR gene and/or another gene in the RP3 region have been suggested as possibilities for the relatively low frequency of observed mutations.

RPGR transcripts appear to be present in all tissues, although expression levels were barely detectable in retina and retinal pigment epithelium, thought to be the primary sites of disease manifestation in XLRP. The putative RPGR gene product of 815 amino acids contains six tandem repeats that show a high degree of homology to the RCC1 protein, a GEF for RanGTPase (9). It was, therefore, proposed that the RPGR gene product functions as a GEF for Ran or a Ran-like protein in the retina or retinal pigment epithelium (6–8). To investigate the physiological function of the RPGR protein and to gain insights into the biochemical mechanism of retinal degeneration caused by RPGR mutations, we have cloned and characterized the full-length and variant cDNA isoforms derived from the mouse homolog of the human RPGR gene (designated mRpgr). In addition to describing several novel features, we demonstrate that the mRpgr protein is isoprenylated and is localized primarily in the Golgi complex.

EXPERIMENTAL PROCEDURES

Isolation of cDNAs and Sequence Analysis—Methods used for routine recombinant DNA analysis were essentially as described (10, 11). Oligonucleotide primers used for sequencing and PCR were synthesized at the Medical School Biomedical Research Core Facility or at NBI/Genovus (Plymouth, MN). A mouse brain cDNA library in pCMV.S-PORT2 plasmid vector was purchased from Life Technologies, Inc. The probe for library screening was derived from a partial human RPGR cDNA (clone C11, spanning exons 2–6; Ref. 6) that was obtained by cloning a PCR product amplified from a human fetal brain cDNA library (FB2; Ref. 12). Hybrid-N membrane (Amersham Pharmacia Biotech) filters containing 30,000–40,000 colonies were hybridized at 42 °C with the 32P-labeled C11 probe in the presence of 37% formamide. DNA from purified positive clones was characterized initially by restriction enzyme analysis. Nucleotide sequence of the cDNA clones was determined by the dideoxy chain termination method using Sequenase™ (U. S. Biochemical Corp.). Nested oligonucleotide primers were used to obtain the complete sequence of both strands. Analyses of the cDNA and derivative polypeptide sequences were performed with MacVector and GCG software (Oxford Molecular Group, Inc.).

Southern Hybridization to Mouse Otc Region YAC Clones—A YAC

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contig spanning the mouse Otc gene was assembled by determining the location of the first and last exons of Otc within each YAC-cloned DNA fragment and by placing the terminal sequences of each YAC relative to the others in the contig. The terminal sequences of each YAC were isolated by established techniques (13) and oriented on the contig relative to the others in the contig. The terminal sequences of each YAC were placed by hybridization to EcoRI-digested total yeast strain DNA. For mapping the mRprg gene, total yeast DNA (2 μg) from four Otc YAC strains (OT1, OT3, OT4, and OT7), an unlinked mouse YAC strain, C41D2, and the yeast host strain, AB1380 (14), was digested with EcoRI. The resulting fragments were separated on a 1% agarose gel and transferred to GeneScreen Plus nylon membrane (NEN Life Science Products) following the manufacturer’s instructions. The hybridization of the mRprg cDNA to the Otc YAC DNA panel was performed according to standard procedures (10).

Expression Analysis—Northern blots of poly(A) + RNA from mouse tissues (MTN blots) were purchased from Clontech (Palo Alto, CA), and hybridization was performed according to the procedure recommended by the manufacturer. The 52P-labeled cDNA probes were generated with random primers (Megaprime kit, Amersham Pharmacia Bio-tech). Quick-CloneTM cDNAs (purchased from Clontech) from adult mouse brain, testis, and kidney were used for PCR amplification with the following primer set: F3 (sense strand, in putative mRprg exon 6), 5′-ATC TCT TGT GGA TAT TAC C-3′; B9 (antisense strand, in putative exon 19), 5′-AGA ACA AAT GTG CTC GGT GAC C-3′. The thermal cycling profile was 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. PCR products were separated in a 1% agarose gel and transferred to a Hybond-N membrane. Southern blots were hybridized with overnight with mRprg-1 cDNA probe, washed at high stringency, and exposed for autoradiography.

Antibodies—A 16-mer synthetic peptide (called GR-P1; GRKSFAEN-NPQKFWPFK) from the amino-terminal region of the deduced human RPRG protein (6) was used to raise antisera in rabbits, and anti-RPRG antibodies were affinity purified by elution from a peptide-conjugated column (Research Genetics, Huntsville, AL). The sequence of the GR-P1 peptide is similar to its corresponding region in the mRprg cDNA (14/16 identical residues; see Fig. 2). The mouse anti-a-mannosidase II monoclonal antibody (53FC3) was obtained from Berkeley Antibody Co. (Richmond, CA). Texas red-conjugated affinity-purified goat anti-mouse F(ab′)2 IgG was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY), resuspended in 50% glycerol, and stored at −20 °C. Affinity-isolated goat anti-rabbit IgG conjugated to fluorescein isothiocyanate was obtained from Sigma.

In Vitro Translation and Immunoprecipitation—The mRprg cDNAs cloned in pCMVSPORT2 vector contain a SP6 promoter in the 5′-upstream region. Protein products were translated in vitro using the TNT SP6 Quick Coupled Transcription/Translation system (Promega), as recommended by the manufacturer. For some of the experiments, in vitro translated mRprg proteins were immunoprecipitated with anti-RPRG antibody (1:200 dilution) by overnight incubation at 4 °C in presence of protease inhibitors (Pharmigen). The immunoprecipitate was collected with protein A-Sepharose (Amersham Pharmacia Biotech) and washed successively with phosphate-buffered saline containing 0.1% and 0.05% Triton X-100 in presence of 20% normal goat serum for 30 min. The rabbit anti-RPRG and the mouse a-mannosidase II antibodies were used for immunoprecipitation of labeled mRpgr products as described in a previous section. At 48 h post-transfection, cells were subjected to immunoprecipitation with appropriate antibody. The immunoprecipitate was analyzed by SDS-PAGE. The gel was treated with dimethyl sulfoxide/2,5-diphenyloxazole and visualized by fluorography (15).

Indirect Immunofluorescence—COS cells were grown overnight on glass coverslips and transfected with DNA from expression constructs as described in a previous section. At 48 h post-transfection, cells were processed for immunofluorescence studies (19). In brief, the cells were washed in phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 in presence of 20% normal goat serum for 30 min. The rabbit anti-RPRG and the mouse a-mannosidase II antibodies were used for a dilution of 1:200. The secondary fluorescein isothiocyanate-conjugated anti-rabbit and/or Texas red anti-mouse antibodies were applied at a dilution of 1:80 and 1:50, respectively. Where indicated, brefeldin A (2.5 μg/ml) was included 1 h, or mevastatin (20 μM) 12 h, before the fixation of cells.

RESULTS

Isolation and Sequence Analysis of the mRprg cDNAs—A 437-bp fragment (C11) of the human RPRG cDNA, spanning exons 3–6 (see “Experimental Procedures”), was used to screen ~700,000 independent clones of an amplified mouse brain cDNA library. Three positive clones, Mcgr16, Mcgr21, and Mcgr29, which resulted from this screening, contained inserts of 2, 2.6, and 2.7 kb, respectively. Sequence analysis of the three clones revealed that Mcgr29 represented the full-length transcript (designated mRprg-1), whereas Mcgr21 and Mcgr16 (called mRprg-2 and mRprg-3, respectively) were derived from variant isoforms (see below). The complete nucleotide sequence of the mRprg-1 cDNA (2,729 bp) revealed two putative in-frame initiation codons (ATG) that precede a long open reading frame. Based on transient expression studies, it appears that both ATGs (referred to as Met-1 and Met-39) are utilized yielding a single amino acid substitution of cysteine 743 to glycine was introduced at position 2, using a Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) per the manufacturer’s instructions. In addition, a single amino acid substitution of cysteine 743 to glycine was introduced in pEDM1 to generate the pEDM1CTG construct. The sequence strand of primers used for site-directed mutagenesis is listed below (mutated residue is underlined): ATG1 (nucleotides 6–37), 5′-GCA CAGTATGCTGGTATCCAAAGAGTGTCG-3′; ATG2 (nucleotides 122–152), 5′-GGGACGGCTCCGCGTGCGAGTAATC-3′; 5′-CAGTGAATGGCTCCGCATCCGTCGCTCTGGTATGGTCTCGG-3′; ATG3 (nucleotides 122–152), 5′-GGGACGGCTCCGCGTGCGAGTAATC-3′; 5′-CAGTGAATGGCTCCGCATCCGTCGCTCTGGTATGGTCTCGG-3′; ATGAAATGC-3′. pED-ra1B was generated by subcloning the AS181 cDNA for small GTP-binding protein ra1B (17) into the pED expression plasmid. All constructs were checked by sequencing of both the DNA strands.

Transfection and Transient Expression Studies—COS cells were grown in six-well culture plates (50–50% confluent) and transfected with 8 μg of DNA from pED expression constructs using the DEAE-dextran method (11, 15). At 36 h post-transfection, cells were starved in methionine-deficient Dulbecco’s modified Eagle’s medium for 30 min and then reconstituted in Dulbecco’s modified Eagle’s medium containing [35S]methionine (50 μCi/ml, 1,000 Ci/mmol, Amersham Pharmacia Biotech). After a 3-min incubation at 37 °C, the cells were incubated in Dulbecco’s modified Eagle’s medium containing 20 μM mevastatin for 3 h. This was followed by a 12-h incubation at 37 °C with [3H]mevalonolactone (50 μCi/ml, 33 Ci/mmol; from NEN Life Science Products) in the presence of 20 μM mevastatin. After washing three times with phosphate-buffered saline, attached cells were solubilized in Nonidet P-40 lysis buffer (200 μl) containing protease inhibitor mixture. The supernatant, collected after a 5-min centrifugation at 3,000 rpm, was subjected to immunoprecipitation with appropriate antibody. The immunoprecipitate was analyzed by SDS-PAGE. The gel was treated with dimethyl sulfoxide/2,5-diphenyloxazole and visualized by fluorography (15).

Isolation and Sequence Analysis of the mRprg cDNAs—A 437-bp fragment (C11) of the human RPRG cDNA, spanning exons 3–6 (see “Experimental Procedures”), was used to screen ~700,000 independent clones of an amplified mouse brain cDNA library. Three positive clones, Mcgr16, Mcgr21, and Mcgr29, which resulted from this screening, contained inserts of 2, 2.6, and 2.7 kb, respectively. Sequence analysis of the three clones revealed that Mcgr29 represented the full-length transcript (designated mRprg-1), whereas Mcgr21 and Mcgr16 (called mRprg-2 and mRprg-3, respectively) were derived from variant isoforms (see below). The complete nucleotide sequence of the mRprg-1 cDNA (2,729 bp) revealed two putative in-frame initiation codons (ATG) that precede a long open reading frame. Based on transient expression studies, it appears that both ATGs (referred to as Met-1 and Met-39) are utilized yielding polypeptides of 746 (calculated molecular mass, 82.2 kDa) and 742 amino acids (calculated molecular mass, 81.7 kDa) respectively. The amino-terminal region of the two deduced mRprg-1 protein isoforms includes six complete tandem repeats (similar to those present in the human RPRG protein, see Ref. 6), which show homology to repeats identified in BCC1, a gunanine nucleotide- exchange factor for Ran-GTPase (9). Secondary structure analysis demonstrates a high degree of hydrophilicity of the
charged carboxyl-terminal region; nevertheless, a specific iso-
preneylation site is present at the carboxyl terminus (CTIL) (20, 21). Seven potential N-glycosylation sites (N-X-S/T), a
number of consensus sequences for S(T)-linked O-glycosylation
(22), and phosphorylation sites for several protein kinases can
be identified in the mRpgr-1 protein(s). At the 3'-end, the
nucleotide sequence contains a poly(A) tract that begins 25 bp
downstream of the polyadenylation signal, AATAAA. As in the
human sequence, ATTTA motifs are also present in the 3'
untranslated region of the mRpgr-1 cDNA.

Comparison of the mRpgr-1 sequence with those of mRpgr-2
and mRpgr-3 revealed that the 5' and 3'-ends of the three
cloned begin and end within few bp of each other (data not
shown). Nevertheless, the two shorter cDNAs contain deletion(s)
of internal sequence regions. Based on a comparison with the
 exon structure of the human RPGR cDNA (6), the internal deletions in mRpgr-2 and mRpgr-3 appear to be distinct exons, i.e. exons 18 and 19a in mRpgr-2, and exons
8–11 and 18 in mRpgr-3 (shown schematically in Fig. 1). The
mRpgr-2 cDNA has an in-frame deletion of 120 nucleotides (bp
1966–2085) and is predicted to encode a polypeptide of 706 or
668 amino acids, depending upon the initiation codon utilized.
The mRpgr-3 cDNA has two deletions; the first spans 630
nucleotides from bp 913 to 1542, and the second is of 89 nucleotides from bp 1966 to 2054. Although the first deletion in mRpgr-3 does not alter the reading frame, the second deletion
carries a frameshift that would result in a truncated protein of
442 or 404 amino acids.

Sequence comparison revealed that the initiation codon of the
reported human RPGR gene product corresponds to the second
ATG codon (Met-39) in the mRpgr-1 cDNA. The deduced mouse and human RPGR proteins share a high degree of sequence homology (64% identity); however, the RCC1 homology region of the two proteins has 80% identical residues (Fig. 2). The two GTP phosphate binding motifs (23) in the amino-terminal region and the hydrophobic domain including the isoprenylation site at the carboxyl terminus are also conserved
in the mRpgr-1 protein. However, the charged domain of the mouse protein is shorter in length by 107 amino acids and shows
a relatively low sequence identity (40%) with the human protein.

Localization of the mRpgr Gene Relative to Otc—Because of
significant divergence in the nucleotide sequence of the human
and mouse cDNAs, we wished to confirm that the mouse ortholog of the human RPGR gene has indeed been identified.
Hybridization of a Southern blot of mouse genomic DNA, di-
gested with different restriction enzymes, with mRpgr cDNA
detected several distinct bands (nine bands in EcoRI digest;
data not shown); however, the results were consistent with a
single copy gene of 50–60 kb (similar to that of human; Ref. 6).
Because the human RP3 locus is linked to OTC at Xp21.1, it
was anticipated that mRpgr might be within the Otc-region
YAC contig. Otc is located on the proximal mouse X chromosome, approximately 4 cm from the centromere. To localize the
mRpgr gene, the mRpgr-3 cDNA was hybridized to a Southern
blot of four YACs (OT1, OT3, OT4, and OT7) from the Otc
contig (Fig. 3A). The mRpgr-3 cDNA hybridized to nine EcoRI fragments (ranging from 1 to 8 kb) in three of the Otc YAC clones (OT3, OT4, and OT7) and to five fragments in another
YAC (OT1) (Fig. 3B). No signal was detected in either the host
yeast strain or an unlinked mouse YAC, C41D2 (used as control).
Because all mRpgr hybridization signals are present in
YAC OT3, it can be inferred that the majority of the mRpgr
gene is contained on this YAC. The distal limit of the mRpgr
interval is defined by the chimeric breakpoint of OT7, between
Otc exons 6 and 7. The location of the mRpgr gene near Otc
was confirmed by hybridization with a rare-cutter restriction en-
zyme digestion panel of YAC OT4. The hybridization pattern of
the cDNA was consistent with previous mapping information and
places mRpgr within 50 kb 5' (proximal) to the Otc gene
data not shown). The mRpgr 5'-region is spanned by YAC
OT3, in agreement with the initial placement of mRpgr on the
YAC contig map.

Expression of mRpgr—To determine the pattern of mRpgr
expression and the size of full-length transcript(s), Northern
blots of poly(A) RNA from mouse embryos and adult mouse
tissues were hybridized with mRpgr cDNA probes (Fig. 4A).
During embryonic development, a major 2.7-kb mRpgr RNA is
present at all of the stages examined and can be detected as
early as 7th day of gestation (see lanes 1–4). In the adult, mRpgr
appears to be expressed with a high level of expression in the
brain and testis (lanes 6 and 12). Another larger transcript of 2.8 kb can also be detected in the brain at shorter exposures (data not shown). Two mRpgr transcripts of
3 and 3.3 kb are observed in the testis RNA (see lane 12).
To investigate the presence of alternatively spliced isoforms,
an mRpgr primer set (F3 and B9, see “Experimental Proce-
dures”) flanking the deletions in mRpgr-2 and -3 cDNAs was
used to amplify products from the reverse transcribed cDNAs of
adult mouse brain, kidney, and testis. Southern blots of ampli-
fied products were hybridized with the mRpgr-1 cDNA probe
(Fig. 4B). In mRpgr-1, the product of PCR amplification with
F3 and B9 primers is 1,578 bp. Two major products of 1.6 and
1.4 kb, corresponding to the mRpgr-1 and mRpgr-2 cDNA,
respectively, were detected in brain, testis, and kidney. A mi-
nor product of about 1 kb (probably corresponding to mRpgr-3)
was also observed. In testis, two additional products of 1.9 and
2.2 kb were detected, confirming the presence of larger mRpgr
transcripts revealed by Northern analysis.

Aberrant Mobility of mRpgr Protein(s) in SDS-PAGE Is Pri-
marily the Result of the Charged Domain—

To examine the encoded protein product(s), mRpgr cDNAs were used as tem-
plate for in vitro translation in a reticulocyte lysate system.

Affinity-purified anti-RPGR antibodies that specifically immu-
oprecipitated the mRpgr protein from transfected COS cell
extracts (see below) were used to immunoprecipitate mRpgr
products from the translation reaction. In vitro translated
products were larger than the calculated molecular mass; two
bands of approximately 110 and 80 kDa were detected as mR-
pgr-1 products with slightly smaller size proteins with mR-
pgr-2, whereas translation of the mRpgr-3 cDNA produced a
protein of 60–62 kDa (data not shown).

The apparent higher molecular mass of the mRpgr protein(s)
can be attributed either to post-translational modifications or
aberrant mobility on SDS-PAGE. This prompted us to evaluate
these findings in a mammalian expression system. The mRpgr
expression constructs used in this study for transient transfec-
tion of COS cells are shown in Fig. 5. The expression of mRpgr
proteins in transfected COS cells was examined by metabolic
labeling with35S-methionine followed by immunoprecipitation
and SDS-PAGE (Fig. 6A). A 110-kDa protein band was ob-
served for mRpgr-1, whereas the two splice variants mRpgr-2
and mRpgr-3 resulted in 105-kDa and 60-kDa proteins, re-
spectively (lanes 2–4). There was no detectable immunoprecipi-
tated protein in the mock-transfected samples (see lane 1).

The size of resulting mRpgr proteins in transient expression studies
matched closely to that obtained from the in vitro translation
experiments.

Because there was a considerable difference in the observed and
calculated molecular masses, we wanted to examine whether the unusually large charged domain in the mRpgr
proteins is responsible for their aberrant mobility on SDS-
polyacrylamide gels. The results presented in Fig. 6A (lanes
5–7) showed that expression constructs that deleted the
charged domain produced proteins of relative molecular mass
closer to the calculated molecular mass (for details, see Fig. 6A
legend).

Both In-frame Initiation Codons Are Utilized Efficiently in
COS Cells—Interestingly, the transient expression studies also
revealed two protein products from several of the expression
plasmids (the products from pEDmR1 and pEDmR2 were not
resolved because of the large size) (see Fig. 6A, lanes 4–7).
We hypothesized that these species may be products of two differ-
ent initiation codons (Met-1 and Met-39, see Fig. 2) that are
utilized efficiently in COS cells. To investigate this, the two
initiation codons were mutagenized to a valine codon in expres-
sion constructs and examined separately for the mRpgr pro-
teins in transfected COS cells (Fig. 6B). The results demon-
strate that the two species are indeed produced from Met-1 and
Met-39 codons.

The mRpgr Protein Is Isoprenylated—Although the mRpgr
protein contains repeats similar to RCC1, it has a highly con-
served isoprenylation sequence at the carboxyl terminus
(-CTIL), which is generally present in small GTP-binding pro-
teins and is required for their proper localization (24). To ex-
amine if the mRpgr protein is isoprenylated at the carboxyl
terminus, we performed metabolic labeling experiments in
transiently transfected COS cells. The results shown in Fig. 7
demonstrate the incorporation of [3H]mevalonate, a precursor
of isoprenoid moiety, in the full-length mRpgr protein (lane 2)
and in the mRpgr protein containing an internal deletion of the
charged domain (lane 3). However, mevalonate incorporation is
not observed when the C residue in the -CTIL sequence is
replaced by G (lane 4). The level of expression in COS cells
was similar for both native and mutated mRpgr proteins, as deter-
mined by immunoprecipitation of 35S-labeled protein in paral-
lel experiments (data not shown). An expression construct with
the small GTP-binding protein ralB, which contains a similar
CTIL sequence at the carboxyl terminus (17) and is shown to be geranylgeranylated (25), was used as a control for isoprenylation (lane 5).

**mRpgr Is Localized in the Golgi Complex**—Because of its homology with RCC1 (9), the mRpgr protein has been proposed to function as a GEF for Ran-GTPase (or a Ran-like protein), a small GTP-binding protein essential for nuclear transport (26). Subcellular localization of the mRpgr protein, therefore, is critical for deciphering its physiological function in vivo. Immuno-
obscured in cells treated with mevastatin, a specific hydroxymethylglutaryl-CoA reductase inhibitor that is known to block prenyl modification of mammalian proteins (Fig. 8, panel E). The use of expression constructs containing different deletions in the mRpgr protein demonstrated the requirement of the carboxyl-terminal -CTIL sequence for specific localization to Golgi (Fig. 8, panels F–H). More importantly, a single amino acid replacement of cysteine 743 in the conserved -CTIL sequence by glycine disrupted Golgi distribution of the mRpgr protein (Fig. 8, panel I). Because of extremely low levels of Rpgr expression in native COS cells, no staining was detected in mock-transfected cells (data not shown).

DISCUSSION

To investigate physiological function of the RPGR protein and to generate a mouse model of X-linked RP, we have isolated and characterized the mouse homolog of the RPGR gene, designated as mRpgr. Sequence analyses of three independent mRpgr cDNAs have revealed several novel features that will be valuable in determining the function of the Rpgr protein. The mRpgr-1 cDNA represents a full-length transcript because (i) it is similar to the size of the major Rpgr mRNA observed on Northern blots; (ii) it contains a poly(A) tail at the 3′-end; and (iii) its comparison with the mouse genomic DNA sequence reveals appropriate context for initiation of transcription.2 Significant divergence in the nucleotide sequence, particularly in the putative exon 1 and in exons 11–17, forced us to map the mRpgr gene physically. In humans, the first exon of RPGR was localized within 25 kb of the first exon of OTC (6). The region surrounding Otc has been investigated extensively as to its evolutionary conservation between human and mouse (30). Because the region has undergone little rearrangement between the two species, it was expected that mRpgr would be located near the mouse Otc gene. The mapping of the mRpgr gene, in complete physical synteny with the human gene, confirmed that we have indeed isolated the mouse ortholog of the human RPGR gene.

Comparison of the human and mouse Rpgr protein sequences argues for a significant function for the conserved RCC1 homology repeats and may have strengthened the proposed function of Rpgr as a GEF for a small GTP-binding protein(s). However, the recently published crystal structure of RCC1 (31) reveals that the seven conserved repeats serve as structural units and form seven blades of a propeller structure and that only one of the residues apparently needed for GEF function of RCC1 is conserved in the Rpgr protein. Interestingly, a large region in the middle (encoded by putative exons 11–17) of the Rpgr protein does not appear to be conserved between human and mouse at the level of primary amino acid sequence. Nevertheless, this part, although smaller by 107 residues in the mouse protein(s), is rich in charged and polar residues (48/196 acidic amino acids) and may provide a hydrophilic and helical interaction domain. Unlike most GEFs, the Rpgr protein possesses a specific isoprenylation signal (CTIL) at its carboxyl terminus, suggesting that the protein may be anchored to a membrane. Several such unique properties of the Rpgr protein suggest that the subcellular localization of the Rpgr protein would be crucial for determining its physiological function.

The mRpgr-2 and mRpgr-3 cDNAs appear to represent naturally occurring truncated isoforms derived from the mRpgr gene. The functional implications of a small in-frame deletion in the mRpgr-2 protein are not obvious. However, the deduced mRpgr-3 protein(s) would not contain two of the RCC1 homology repeats and the carboxyl-terminal region including the potential isoprenylation site and is predicted to have an altered localization and functional properties. Our results provide evidence of variant Rpgr isoforms in different tissues, although no attempt was made to deduce relative levels of different Rpgr transcripts. Detection of transcripts during early embryonic development (at E7, before the differentiation of optic cup begins) in mouse would imply a significant role for the Rpgr protein in cellular differentiation. However, null mutations in the RPGR gene result in only photoreceptor degeneration (8, 32, 33). This is reminiscent of another X-linked retinal disease, choroideremia, where the CHM gene product (REP1) is a component of Rab geranylgeranyl transferase (34). It has been suggested that in most cells the REP1 function can be replaced by another homologous protein (REP2), whereas its absence leads to deficient geranylation of Rab 27, expressed in the retinal pigment epithelium and choriocapillaris (35). Our analysis indicates that some of the Rpgr transcripts may be expressed differentially, as in case of testis. It is possible that the Rpgr function is redundant in most cells but is needed in the photoreceptors, retinal pigment epithelium, and/or neighboring cells. Interestingly, sperm abnormalities have been noted in certain families with XLRP (36); whether this is related to larger RPGR variants expressed in testes remains to be determined.

The availability of a full-length mouse cDNA has allowed us to perform protein expression studies. An interesting finding is the identification of two in-frame initiation codons (Met-1 and Met-39) in mRpgr cDNAs. Initially, it was difficult to deduce which of the two codons is preferentially used for translation of the mRpgr protein in vivo. Although the first ATG is generally utilized for translation, the second ATG resides in a better consensus sequence region (GCCPuCCATGG; 37) and corresponds to the initiation codon in the reported human cDNA (6). The transient expression studies demonstrate that both ATGs are functional and utilized efficiently at least in COS cells. The physiological implication of 38 additional residues at the amino terminus of the mRpgr protein (generated with Met-1) is not clear at this stage. We have also established that higher than calculated molecular mass products (i.e., observed retarded mobility on SDS-PAGE) produced by the mRpgr cDNAs are a consequence of secondary structure of the mRpgr protein(s) resulting from the presence of an unusually large charged and polar domain and clustering of glutamic acid residues. A slight increase in observed molecular size can also be accounted by predicted post-translational modifications.

The data presented in this report demonstrate that the mRpgr protein has two important properties: (i) It is isoprenylated at the carboxyl terminus. This is generally a characteristic of small GTP-binding proteins; Ras proteins are farnesylated because X is a non-leucine residue in the carboxyl-terminal -CAAX sequence, whereas Rho and Rab proteins are gera-

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2 R. Fujita, C. Chen, and A. Swaroop, unpublished data.
nylgeranylated because of a leucine at the carboxyl terminus (24). Because of a -CTIL sequence, it is hypothesized that the mRpgr protein is geranylgeranylated. (ii) The mRpgr protein is primarily localized in the Golgi complex, instead of the nucleus where RCC1 is detected. Although our results do not exclude the presence of the mRpgr protein in the cytoplasm, this pattern of subcellular distribution provides evidence against mRpgr’s involvement in nuclear transport as a GEF for Ran or a Ran-like protein. It can be argued that overexpression of mRpgr in transiently transfected COS cells may have altered its intracellular distribution; however, our preliminary retinal subcellular fractionation studies are also consistent with the Golgi and cytosolic distribution of mRpgr. We, therefore, propose that the mRpgr protein is involved in transport process at the Golgi. It should be noted that many small G-proteins are involved in transport of rhodopsin to photoreceptor outer segments (38) and that defective rhodopsin transport has been shown to be the underlying defect in many mutations in autosomal dominant RP (39–41). In addition, a Ran-binding protein, RanBP2, has been implicated previously in the transport of cone opsins (42). Further investigations are in progress to examine the role of mRpgr in rhodopsin transport. One of the major questions that we wish to address is how mutations in RPGR lead to photoreceptor degeneration. The characterization of mRpgr cDNAs represents a significant step for developing a mouse model of XLRP by knockout strategy. These studies also provide novel insights into Rpgr function and form the basis for further investigations needed to elucidate the molecular mechanisms of XLRP pathogenesis.

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