Limited proteolysis differentially modulates the stability and subcellular localization of domains of RPGRIP1 that are distinctly affected by mutations in Leber’s congenital amaurosis

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The retinitis pigmentosa GTPase regulator (RPGR) protein interacts with the retinitis pigmentosa GTPase regulator interacting protein-1 (RPGRIP1). Genetic lesions in the cognate genes lead to distinct and severe human retinal dystrophies. The biological role of these proteins in retinal function and pathogenesis of retinal diseases is elusive. Here, we present the first physiological assay of the role of RPGRIP1 and mutations therein. We found that the monoallelic and homozygous mutations, ΔE1279 and D1114G, in the RPGR-interacting domain (RID) of RPGRIP1, enhance and abolish, respectively, its interaction in vivo with RPGR without affecting the stability of RID. In contrast to RIDWT and RIDD1114G, chemical genetics shows that the interaction of RIDΔE1279 with RPGR is resistant to various stress treatments such as osmotic, pH and heat-shock stimuli. Hence, RIDD1114G and RIDΔE1279 constitute loss- and gain-of-function mutations. Moreover, we find that the isoforms, bRPGRIP1 and bRPGRIP1b, undergo limited proteolysis constitutively in vivo in the cytoplasm compartment. This leads to the relocation and accumulation of a small and stable N-terminal domain of ∼7 kDa to the nucleus, whereas the cytosolic C-terminal domain of RPGRIP1 is degraded and short-lived. The RIDD1114G and RIDΔE1279 mutations exhibit strong cis-acting and antagonistic biological effects on the nuclear relocation, subcellular distribution and proteolytic cleavage of RPGRIP1 and/or domains thereof. These data support distinct and spatiotemporal subcellular-specific roles to RPGRIP1. A novel RPGRIP1-mediated nucleocytoplasmic crosstalk and transport pathway regulated by RID, and hence by RPGR, emerges with implications in the molecular pathogenesis of retinopathies, and a model to other diseases.

INTRODUCTION

Retinitis pigmentosa is a genetically heterogeneous disease for which approximately 32 loci have been identified (RetNet Web site). X-linked retinitis pigmentosa type 3 (XIRP3) (MIM 312610) accounts for up to 13% of all RP cases in North America (1). Subsets of genetic lesions in this locus have been implicated also in other retinal dystrophies such as cone (2) and cone–rod (3) dystrophies and macular degeneration (4). Recently, some mutations in XIRP3 were linked to non-ocular and systemic diseases such as progressive hearing loss, increased susceptibility to upper respiratory and sinus infections (5–7). The XIRP3 locus encodes at least two major protein isoforms derived by alternative splicing (8–10). These share an N-terminal domain homologous to RCC1, the guanine-nucleotide exchange factor of RanGTPase (11), and a variable C-terminal domain containing either an isoprenylation site (12) or an acidic domain derived from the retention of the terminal intron 15 (10). In light of the homology of these proteins isoforms to RCC1, the protein isoforms encoded by XIRP3 were named retinitis pigmentosa GTPase regulator (8).
In an effort to understand the molecular pathogenesis of XRIP3, the retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIP1) was identified from a genetic yeast two-hybrid screen as a molecular partner of retinitis pigmentosa GTPase regulator (RPGR) (13,14), a finding subsequently confirmed by others (15,16). This interaction is mediated between the RCC1-homologous domain (RHD) of RPGR and the C-terminal domain, RPGR-interacting domain (RID), of RPGRIP1 (13). RPGR and RPGRIP1 associate in vitro and in vivo and they partially colocalize to the outer segment compartment of photoreceptors (13,17). Point mutations in the RHD of RPGR abolish the interaction with the RID of RPGRIP1 (13). The human, bovine and murine loci encode several RPGRIP1 isoforms (13,18) and some of these exhibit species-specific expression and subcellular localization (17–19). Moreover, RPGRIP1 is expressed in various inner retinal neurons (17–19), whereas RPGR expression seems mostly restricted to the photoreceptors (13,17).

Mutation analysis of RPGRIP1 in the human linked this locus to Leber congenital amaurosis (LCA) (LCA, MIM 204000) (20–22), the leading cause of blindness in children and a disease typically associated with extinguished ERG during the first years of life (23). Although the broader expression of RPGRIP1 may underlie the phenotypic expression of allied mutations, the molecular basis of the biological role of RPGRIP1 and RPGR, the molecular implications of the interaction between these, and the effect of mutations therein in pathogenic pathways, remain largely elusive. We have previously reported that RPGRIP1 purified from the outer segment compartment of photoreceptor neurons is extremely susceptible to limited proteolysis upon sequential extraction with various detergents (19). In addition, different RPGRIP1 isoforms and/or proteolytic products thereof were differentially sequestered in subcellular compartments of retinal neurons (19). Finally, antibodies against different domains of RPGRIP1 detect distinct RPGRIP1 isoforms in the retina and some of which have species-specific expression (19). For example, an antibody against the conserved RID of RPGRIP1 (e.g. Ab38) recognizes specifically multiple RPGRIP1 isoforms, whereas another against the upstream coiled coil (SMC/CC) domain detects a single and major RPGRIP1 isoform of ~175/150 kDa (19).

Altogether, these data suggest that RPGRIP1 may undergo limited and differential proteolysis in subclasses of retinal neurons. However, biological evidence supporting these observations is lacking. This would be highly relevant for establishing the role of RPGRIP1, RPGR, the binary complex and mutations therein, in the function of these and allied pathogenic molecular processes. To this end, we provide here evidence of limited proteolysis in vivo of RPGRIP1 leading to the production of novel domains with unique biological properties, the molecular basis of a subset of RPGRIP1 mutations in this process and the effect of these on the interactions with RPGR. A model emerges for novel roles of RPGRIP1 in neuronal class and subcellular-specific function, and disease pathogenesis. This model sets the basis for novel biological and pharmacological assays to understand the molecular pathogenesis of retinopathies linked to RPGRIP1, RPGR and molecular partners of these.

RESULTS

The human mutations in the RID of RPGRIP1, ΔE1279 and D1114G, are gain- and loss-of-function mutations

The ΔE1279 mutation is located at the end of the RID of RPGRIP1 and until this date it was found only in a monoallelic state (21,22). Moreover, the ΔE1279 mutation appears to exhibit variable genetic penetrance since its phenotypic expressivity does not always correlate with LCA or any other retinal disease in the human (21). In contrast, the D1114G mutation has been found in homozygous and compound heterozygous states in the human and it always cosegregates with LCA (21,22). To understand the molecular basis of these genetic and clinical observations, we employed the yeast interaction trap assay (14) to determine qualitatively and quantitatively the strength of the interaction in vivo between the RHD of RPGR and the RID of RPGRIP1 and mutations therein. Yeast cells coexpressing the RHD of RPGR with RIDWT, RIDD1114G and RIDΔE1279 of RPGRIP1 were spotted in serial dilutions on solid and selective yeast media. The growth and the colony size (growth rates) of the spotted yeast cells were monitored for ~3–4 days. As shown in Figure 1A, in non-selective media, none of the constructs employed was toxic and/or affected yeast growth, their growth rates and colony morphology (Fig. 1A, left). In contrast, in selective media, the RIDΔE1279 construct exhibited faster and stronger growth than its counterpart constructs, RIDWT and RIDD1114G (Fig. 1A, right). No growth was observed for RIDD1114G (Fig. 1A, right). Quantitative growth assays were carried out with the same constructs (Fig. 1B). These corroborated the solid growth assays. To this end, the RIDΔE1279 construct had ~35% stronger interaction with RPGR than the wild-type construct, whereas the RIDD1114G construct exhibited ~95% decrease in the strength of the interaction with RPGR. To determine whether these effects were caused indirectly by a change in vivo in protein stability in yeast that was mutation-dependent rather than by a direct effect on the interaction between RPGRIP1 and RPGR, we carried out western blot analysis with yeast extracts coexpressing the constructs described (Fig. 1C). To this end, the expression levels of the fusion constructs employed were similar, thus supporting a direct effect of the mutations in affecting the formation of the binary complex mediated between the interacting domains of RPGR and RPGRIP1.

A chemical genetic (24,25) approach with transformed yeast cells was employed to probe further the interaction between RPGR as well as RIDΔE1279 and RIDD1114G (Fig. 2). Among the treatments employed, we found that lowering the growth temperature of yeast cells transformed with RIDWT and RIDΔE1279 to 22°C led to comparable growth of these, whereas this temperature had no effect on the interaction between RIDD1114G and RPGR. We took advantage of the normalizing effect of the restrictive temperature phenotype between the RIDWT and RIDΔE1279 constructs to investigate the strength in vivo of the interaction between these and RPGR upon stress stimuli. Among the several compounds tested, we found that sorbitol (osmotic stress), heat-shock (chaperone-induction) and the pH reduction of the media to 4.5 (change in protein overall charge) always lead to a significant decrease of the interaction between RPGR and RIDWT,
while increasing or maintaining that with RID^{ΔE1279}. Treatment with dimethyl sulfoxide (DMSO) (chemical chaperone) has an increased effect on the interaction of RIDWT, but not of RID^{D1114G}. None of these treatments has any effect on RID^{D1114G}. Altogether, these results support an increased affinity between RPGR and RID^{ΔE1279} that is resilient to physicochemical stimuli under various chemical and physical treatments.

**RPGRIP1 isoforms and domains thereof localize differentially among subcellular compartments in transfected and cultured cells**

We have previously identified a murine-specific RPGRIP1 isoform, mRPGRIP1b, which lacks the C2 and RID domains (Fig. 3A) (18). This isoform is distributed exclusively throughout the cytosol, where it colocalizes partially with a subpopulation of lysosomes in cultured cells and retinal neurons (18). To contrast the subcellular distribution between the three major RPGRIP1 isoforms (Fig. 3A) and to investigate their function, the molecular and phenotypical effect of mutations in the function of these, we carried out quantitative phenotypic analysis of the distribution of bRPGRIP1, bRPGRIP1b and mRPGRIP1b ectopically expressed in cultured COS7 cells, which lack RPGRIP1 expression. Although similar qualitative results to the ones described subsequently were obtained with the neuroretinal lines 661W, which express RPGRIP1 (19), and RGC5 (data not shown), we carried out detailed quantitative phenotypic analysis with COS7 cells in light of their high transfection efficiency. The bRPGRIP1 contains all structural/functional domains, whereas the bRPGRIP1b lacks the C2 domain (Fig. 3A). These isoforms were tagged at the N- and C-terminal ends with EGFP- and the HA-epitopes, respectively, to survey for differences in subcellular localization of the N- and C-terminal domains among the three RPGRIP1 isoforms. As shown in

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**Figure 1.** The ΔE1279 and D1114G genetic lesions in RID of RPGRIP1 are gain- and loss-of-function mutations. (A) Serial-dilution yeast growth assays (30°C) in solid non-selective (left) and selective (right) growth media. None of the constructs exhibited toxicity as surveyed by the growth (and morphology) of yeast colonies in non-selective growth media. In selective media, ΔE1279 mutation exhibited stronger growth and faster growth rates, whereas D1114G failed to grow. (B) GAL4-responsive UAS-his3 reporter growth assay (30°C) in selective liquid growth media of yeast cells coexpressing wild-type RID and mutations therein with RHD of RPGR. The ΔE1279 and D1114G mutations cause, respectively, an increase and decrease of ~35 and 95% of the strength of the interaction between RID and RPGR. Values are the average of three independent experiments and are shown with standard deviation. (C) Western blot analysis of homogenates of ~10^6 yeast cells coexpressing RPGR with the GAL4AD-fused RID constructs. All RID constructs were expressed at similar levels.
The ΔE1279 mutation is resistant to physico-chemical stresses. Serial-dilution yeast growth assays in solid selective growth media at 22°C with the same reporter assay and constructs described in Figure 1 and in the presence of various treatments. Lowering the temperature normalized the growth rates between wild-type and ΔE1279 RID constructs but it did not affect D1114G (left). Treatment with sorbitol, heat-shock pulses and decrease of pH, increased selectively the growth rate of ΔE1279 and it had variable effects on the counterpart wild-type construct. The chemical chaperone, DMSO, led to an increase of the growth rates of wild-type and ΔE1279 constructs. None of the treatments elicited growth of D1114G.

Figure 3, the EGFP-signal was predominantly observed in the nucleus with bRPGRIP1 (Figs 3B–G, K–M and 5A) and bRPGRIP1b (Fig. 4A–I and 5A). In addition, a significant fraction of cells expressing bRPGRIP1 (Figs 3B–D and 5A) and bRPGRIP1b (Figs 4A–C and 5A) had EGFP localized exclusively to and/or in the cytoplasm compartment, too. Among these, bRPGRIP1b-expressing cells represented the lowest fraction of cells with EGFP in the cytosol (Fig. 5B). In contrast, all cells expressing mRPGRIP1b exhibited EGFP-signal only in the cytosol (Fig. 5A). The same observation was found for the C-terminal localization of mRPGRIP1b, which overlapped perfectly with the EGFP-signal (Fig. 5A and B) (18). In contrast, cells expressing bRPGRIP1 and bRPGRIP1b had the HA-signal either singly localized to the cytosol (Figs 3E–G, 4D–F and 5B) or it overlapped with the EGFP when it was present in the cytosol (Figs 3B–D, H–J, 4A–C and 5B). Seldom there were cells with HA-signal in the nucleus (Figs 3, 4 and 5B). Finally, the distribution pattern of RPGRIP1 isoforms among different subcellular compartments was independent of the analysis of the post-transfection times and it was observed as early as 16 h upon transfection (data not shown).

Among the cells with EGFP- and HA-tag expression in the cytosol, two subcellular distribution patterns were observed, reticular and punctuate (Figs 3 and 4). The former distribution pattern was the predominant type observed with EGFP- and HA-tags (Figs 3B–D, H–J and 5C and D), albeit the latter pattern was equally observed with bRPGRIP1b (Figs 4A–C and 5C) and RPGRIP1 (Fig. 5C). Conversely, the cytoplasmic distribution of mRPGRIP1b was consistently punctuate with N- and C-terminal tag reporters (data not shown and Fig. 5C and D). In nuclei, we observed strong and diffuse accumulation of EGFP-signal of bRPGRIP1 and bRPGRIP1b in the nucleolus, predominantly when this signal was absent from the cytosolic compartment (Figs 3E–G, K–M and 4D–I). Whenever the EGFP-signal was localized to the cytosolic compartment, the same signal either was absent from the nucleus (Fig. 3H–J) or was often distributed diffusely (Fig. 3B–D) in the nucleus.

RPGRIP1 is processed proteolytically and constitutively in vivo and the N-terminal domain is relocated to the nucleus

The finding of cells expressing EGFP exclusively in the nucleus, and with this signal compartmentalized from the HA-tag in the cytosol, strongly supports the proteolytically processing in vivo of RPGRIP1 and bRPGRIP1b. This leads to the relocation of the EGFP-fused N-terminal domain to the nuclear compartment and retention and/or degradation of the C-terminal HA-tag and proteolytic processed domain in the cytosolic compartment. To probe further the proteolytic processing of RPGRIP1, we carried out immunoblots of homogenates of cells transfected with RPGRIP1 tagged with EGFP- and HA-epitopes. As shown in Figure 6A, the anti-EGFP Ab detected a major protein of ~34 kDa (EGFP-p7; open arrowhead) and a minor counterpart of ~200 kDa (representing the unprocessed EGFP-fused RPGRIP1, arrow). Other minor proteolytic intermediates were also observed (e.g. filled arrowheads). These were absent in untransfected cells. Hence, the 34 kDa EGFP-fused protein comprises EGFP (27 kDa) and a small N-terminal domain of RPGRIP1 ( ~7 kDa (p7). Conversely, anti-SMC/CC (Fig. 6B, Ab22), anti-RID (Fig. 6C, Ab38) and anti-HA (Fig. 6D) antibodies detected a major protein (arrow) with an apparent molecular weight of ~200 kDa [the apparent molecular mass of unfused bRPGRIP1 is ~175 kDa (19)], which was absent in untransfected cells. Proteolytic products of minor abundance and various molecular masses were detected only with the high-affinity anti-RID antibody (Fig. 6C, filled arrowheads).

We employed a subcellular fractionation approach to investigate the partitioning of bRPGRIP1/bRPGRIP1b, proteolytic products thereof and potential difference of these products between the nuclear and cytosolic compartments. As shown in Figure 6E and F, the unprocessed bRPGRIP1 and bRPGRIP1b and the cleaved N-terminal fragment of bRPGRIP1 (lane 2) and bRPGRIP1b (lane 3) were present in the nuclear (Fig. 6E) and cytosolic (Fig. 6F) compartment fractions. In addition, both bRPGRIP1 and bRPGRIP1b
produced a proteolitically processed N-terminal fragment of identical molecular mass (Fig. 6E and F).

Molecular phenotypes linked to a subset of human mutations in RPGRIP1

The genetic, biochemical and subcellular assays described provided the stage to compare the effect of mutations in RPGRIP1 with the molecular phenotypes described for the wild-type RPGRIP1. For this study, we focused on the RPGRIP1 isoform since this isoform contains all structural/functional domains and seems to be the major isoform in the human and the bovine (but not mouse) retinas (18,19). In addition, we removed the C-terminal tag from RPGRIP1 for this analysis for two reasons. First, in light of the existence of a (monoallelic) mutation at the very C-terminal end of Figure 3.
RPGRI1 (21), we reasoned that a contiguous tag could potentially interfere with the expressivity of its phenotype. Secondly, and in support of this rationale, we found that the C-terminal tag appears to cause an increased retention of RPGRI1 and/or proteolytic products thereof in the cytoplasm, albeit the tags do not affect in any way the localization phenotypes and generation of proteolytic products described for RPGRI1 (data shown subsequently and data not shown). These modifications were also combined with a more stringent survey of the phenotypes scored. These included the localization of EGFP-signal in the nuclear and cytosolic compartments, and in the cytosolic and nuclear compartments alone (Figs 7 and 8). In addition, we surveyed the effect of the mutations on the antigenicity of the SMC/CC domain of RPGRI1 towards antibodies 22 and/or 23 in light of the localization of this domain next to the proteolytically cleaved nuclear domain (and the discovery of novel interacting partners for these domains; data not shown) (Figs 7 and 8). Finally, the effect(s) of the mutations in the generation of RPGRI1-proteolytic intermediates/products was investigated (Fig. 8D). For this study, we focused on three human LCA-mutations shown here and elsewhere to affect the interaction of RPGR with RPGRI1 (13). These are RID\textsuperscript{D1114G}, RID\textsuperscript{DE1279} and a mutation (equivalent to human Q893X) leading to the complete deletion of the RID of RPGRI1, RPGRI1\textsuperscript{ARD} (Fig. 8A) (21).

As shown in Figure 8A and B, all three mutations tested led to dramatic changes in the subcellular distributions of EGFP and SMC/CC of RPGRI1 and antigenicity of RPGRI1 towards its cognate antibodies (Abs22/23). The ΔE1279 (Fig. 7D–I) and ΔRID (Fig. 7M–O), and the D1114G (Fig. 7J–L) mutations lead to a decrease and an increase, respectively, of the localization of the EGFP-tagged N-terminal domain of RPGRI1 in the nuclear and cytosolic compartments (Fig. 8A). In constructs with the D1114G and the ΔRID mutations, extremely few cells were identified expressing EGFP in the cytosolic compartment alone. In ΔRID, this was accompanied by a strong increase of EGFP in the nucleus alone. Conversely, the ΔE1279 caused a significant increase of the N-terminal domain in the cytosolic compartment alone (Fig. 8A). In the nuclear compartment alone, only the ΔRID construct exhibit a significant increase in the nuclear retention of its N-terminal domain (Fig. 8A). The nuclear localization of EGFP in the nucleus was sometimes atypical and characterized by its accumulation in dispersed foci (Fig. 7M–O), in addition to its localization in the nucleolus. The distribution and antigenicity of SMC/CC varied significantly among the constructs tested. The most striking phenotype was observed for the complete lack of antigenicity of SMC/CC towards Abs22/23 in the construct with the D1114G mutation in all subcellular compartments of all cells (Fig. 7J–L). While the SMC/CC was localized mostly to the cytosolic compartment, the ΔRID and the ΔE1279 mutations caused a significant decrease of its localization in this compartment that was accompanied by an increase of similar magnitude of its localization in the nuclear and
cytosolic compartments combined. While extremely few or no cells expressing wild-type RPGRIP1 were observed with nuclear staining alone of SMC/CC, the ∆RID and the ∆E1279 also caused a small but significant increase of nuclear localization alone (Fig. 8B). Noticeably, the SMC/CC never colocalized with EGFP in the nucleus in the mutant constructs, it was always excluded from the nucleolus and its distribution pattern was seen as coarse foci throughout the nucleus (Fig. 7). Finally, EGFP and SMC/CC signals, whenever present, always colocalized in the cytosolic compartment, but the ∆RID construct often exhibited strong accumulation in the perinuclear region. Similar qualitative and phenotypical results found with Ab22 against the SMC/CC domain of RPGRIP1 in this mutation study were also observed for the distribution of RID of RPGRIP1 with an antibody (Ab39) against this domain (data not shown).

To probe the effects of these mutations in the production of proteolytic products and/or intermediates of RPGRIP1, we analyzed immunoblots of homogenates of cells transfected with each mutation construct and incubated with antibodies against EGFP, SMC/CC and RID. As shown in Figure 8C, all constructs tested produced a stable and abundant N-terminal EGFP-fused product of ~34 kDa (EGFP–p7). However, the D1114G construct produced an abundant and stable EGFP-fused product/intermediate of ~45 kDa (EGFP–p17, Fig. 8C, arrowhead) that was never observed for any other constructs. This aberrant 45 kDa EGFP-fused proteolytic product, EGFP–p17, localized in the nuclear and cytoplasmic fractions like that seen for EGFP–p7 (data not shown). In addition, the parent high molecular mass protein was barely detectable when compared with those of other constructs (Fig. 8C, lane 4). Interestingly, the ∆E1279 mutation caused a decrease in the electrophoretic mobility of the unprocessed RPGRIP1. Reprobing of the immunoblot with Abs22 and 38 (against SMC/CC and RID) did not detect any additional differences among the constructs tested in the generation of stable intermediates comprising these domains (data not shown).
DISCUSSION

Mutations in RPGR are associated with several types of severe systemic retinopathies (1,5–7,27), whereas those in RPGRIP1 have been linked only to LCA (20–22), a disease with stronger (and likely broader) retinal expressivity. However, the expression of these phenotypes is observed also for many other genes implicated in retinopathies (1,5,7,21–23,27–31).

Yet, the molecular pathogenesis underlying these phenotypes remains elusive for the most part of the retinal disease-causing genes identified until this date. Like for many human disorders sharing phenotypic expression, the molecular basis of the pathobiological processes leading to the dysfunction, and whenever applicable, death of the disease gene expressing cells, is likely to exhibit strong intra- and interallelic variability. The underlying molecular basis for this
genetic variability is likely to stem in many instances from the variable effect of mutations in distinct structural/functional domains affecting interactions with distinct molecular partners. This, combined with the existence of multiple splice-variants with cell-type and/or species-dependent expression and genetic modifiers altering the disease expression, generates a complex array of distinct biological processes underlying the molecular pathogenesis of diseases. Mutations in RPGR and RPGRIP1 are likely to fit this pathogenic model in light of the mosaic structural architecture of their proteins, strong intra-allelic variability in phenotypes, distinct phenotypes among interacting partners and inter-species variability in isoforms and in expression of disease phenotypes. This report establishes the first biological and well-defined assay to identify molecular, subcellular, biochemical and pathological phenotypes to RPGRIP1 isoforms and mutations affecting the RID domain of RPGRIP1s. It also establishes the framework for assaying the biological effects of interacting partners on RPGRIP1 function and the role of this on their partners and mutations therein.

Figure 7. LCA-linked mutations in RID cause aberrant subcellular localization of RPGRIP1 and domains thereof. Localization of the mutations in RPGRIP1 studied and of the RPGRIP1 antigenic domain of Ab22 are depicted in the diagram shown above the image panels. Cells transfected with the wild-type (A–C), ΔE1279 (D–I) and D1114G (J–L) and ΔRID (equivalent to human Q893X) (M–O) mutations in RID of bRPGRIP1 construct (1.5 µg) tagged at the N-terminal end with EGFP, cause abnormal molecular and subcellular phenotypes. The ΔE1279 (D–I) mutation in RID led to an increased localization of the SMC/CC domain to the nuclear compartment. The SMC/CC domain never colocalized with the EGFP-fused N-terminal domain and it was excluded from the nucleolus (arrow). Moreover, its nuclear distribution pattern (as gross punctuate foci) was distinct from the diffused nuclear distribution of EGFP-fused domain. The D1114G mutation (J–L) in RID completely abolished the immunoreactivity SMC/CC domain towards its cognate antibodies (Ab22/23) in every cell scored and EGFP was often excluded from the nucleolus. The ΔRID (equivalent to human Q893X) mutation (M–O) led to a strong increase of the EGFP-fused N-terminal domain in the nucleus (and decrease from the cytosol), where it was often observed as strong/gross puncta. EGFP (images in the left column) and SMC/CC signals (images in the middle column) are shown, respectively, in green and red. Overlay pictures of these are shown in the right column. Scale bar: 10 µm.
Here, we show that the ΔE1279 mutation causes a significant enhancement of the interaction of RID with RPGR, an interaction highly resistant to stress stimuli. Conversely, the D1114G mutation drastically abolishes this interaction, which is non-responsive to stress stimuli. This provides a molecular rationale for the monoallelic and biallelic (homozygous or heterozygous states) genetic modes found for the ΔE1279 and D1114G mutations (21,22), respectively. It also provides a rationale for the variable genetic penetrance of the ΔE1279 mutation, which is present in carriers and patients harboring this monoallelic mutation (21). The growth of wild-type RID and mutations therein at restrictive temperature suggests that the RID domain is metastable and that the ΔE1279 mutation may stabilize the conformation of RID, hence favoring the interaction with RPGR.

Several RPGRIP1 isoforms have been identified until this date in the retina (13,18,19). In this study, we focused on bRPGRIP1, bRPGRIP1b and mRPGRIP1b isoforms because they contain and/or lack key structural/functional domains.
such as the RID and/or C2 domains. The evidence presented supports that the combination of the C2 and RID domains of RPGRIP1 is necessary for the relocation (and proteolytic cleavage) of the N-terminal domain (ND) to the nucleus. The absence of either C2 (e.g. bRPGRIP1b) or RID (e.g. ΔRID mutation) domains alone does not prevent the nuclear relocation of ND of RPGRIP1 (Figs 3–5). In contrast, the mRPGRIP1b, which lacks the C2 and RID, is completely excluded from the nucleus and is instead partially targeted to a subpopulation of lysosomes (18). On the other hand, exclusion of the C2 domain from RPGRIP1 provides a novel mode of regulation of bRPGRIP1b, since the ND and C-terminal domains are less prevalent in the cytosolic compartment than that observed for RPGRIP1. However, this has no effect on the extent of nuclear localization of these domains.

Analysis of homogenates of COS7 cells transfected with bRPGRIP1 (Fig. 6) supports further that bRPGRIP1 is proteolytically processed into a very stable, abundant and small N-terminal domain (Fig. 6) that becomes competent for nuclear relocation upon cleavage. This contrasts with the parental unprocessed protein, which is of minor abundance (Fig. 6). Conversely, antibodies against any domain downstream of the cleavage site detect predominantly unprocessed bRPGRIP1 (Fig. 6B–D). Minor and smaller proteolytic intermediate products are detected with the high-affinity antibody Ab38 against the C-terminal RID of bRPGRIP1 (Fig. 6C). This supports that the domains downstream of the ND domain are subjected to proteolytic degradation and are short-lived upon cleavage of the ND from bRPGRIP1. The absence singly of the C2 and RID domains do not alter the limited processing of bRPGRIP1, since similar ND fragments of identical molecular masses are produced by limited proteolysis of bRPGRIP1b and ΔRID (Figs 6E and F and 8C). Finally, the data support that the proteolysis of bRPGRIP1/ bRPGRIP1b occurs in the cytosol. To this end, the cleaved ND is observed in the cytoplasmic compartment, the C-terminal fragment is excluded from the nucleus (Fig. 6), there are much fewer EGFP-positive cells with HA-signal (Fig. 5A and B) and the EGFP- and HA-tags colocalize only in the cytosolic compartment (Figs 3 and 4).

The results here presented are in agreement with our observations on the role of the nuclear pore complex (NPC) in inner nuclear neurons, in particular, amacrine neurons (19). In light of RanBP2 interaction with the components of the 19S cap of the proteasome (35), RanBP2 remains a strong candidate for modulating the limited processing of RPGRIP1. Moreover, these results imply the existence of distinct transport mechanism requirements for the mobilization of RPGRIP1 to components of the NPCs (e.g. RanBP2) and nuclear translocation. The removal of putative cytoplasmic localization signals upon processing of the C-terminal region and unmasking of until now elusive nuclear localizing signal (NLS) sequence in ND underlie likely the nuclear import of ND upon processing. To this end, analysis of the N-terminal sequence of RPGRIP1 found the existence of a canonical NLS (KK/RXXR) (36) motif for nuclear import of ND of RPGRIP1. This sequence, K62RLR, is also conserved in the human RPGRIP1 (K62RMR) and its predicted location is at the C-terminal end of the processed ND (Fig. 9). A similar paradigmatic scenario is known to occur for the nuclear trafficking of Ci155 (37) and polycystin-1 (38,39). Ci155 is a cytoplasmic target of Hedgehog signaling pathway (37). In the absence of Hedgehog signaling, Ci155 is proteolytically processed constitutively in the cytosolic compartment to produce a smaller N-terminal fragment (Ci75) that acts as a nuclear repressor of discrete genes (37). Polycystin-1 (PKD1) exhibits cell surface and cytoplasmic localization, and it has been implicated in autosomal dominant polycystic kidney disease (ADPKD). Recently, it was shown that the C-terminal domain of PKD1 is released by proteolysis and that the C-terminal tail is translocated to the nucleus where it activates the AP1-pathway (38).

Finally, we show that the human mutations, ΔE1279, D1114G and ΔRID, lead to phenotypes reflecting dramatic changes in the biological properties identified for RPGRIP1 and proteolytic products thereof. Moreover, these phenotypes are often mutation-specific. This is supported by the observations that the RID loss-of-function mutation, D1114G, causes a redistribution of the processed ND fragment between the nuclear and cytosolic compartments, whereas the ΔE1279 and ΔRID mutations promote its localization in the cytosolic and nuclear compartments alone, respectively (Figs 7 and 8A). In addition, D1114G mutation completely abolishes the immunoreactivity of the upstream domain, SMC/CC towards Abs 22/23 (Fig. 8B), possibly due to an enhancement of its proteolysis and/or production of a pathological and stable intermediate containing a portion of the SMC/CC domain (Fig. 8C). In contrast, the ΔE1279 and ΔRID mutations promote the nuclear relocation of this domain to the nuclear compartment. This proteolytic intermediate does not colocalize with EGFP–ND and it is short-lived since it is not detectable on immunoblots. In addition, there are far less SMC/CC-positive nuclei than those observed for the ND domain. The pathologic accumulation of the ΔRID construct in the perinuclear region (Fig. 7) suggests that the absence of RID deregulates the transport of RPGRIP1 to the nuclear compartment, possibly explaining the pathologic accumulation of ND solely in the nucleus (Fig. 8A). The RID domain of RPGRIP1 is likely to perform multiple roles that are dependent on the presence and/or absence of interacting factors such as RPGR, which is expressed in a subset of retinal RPGRIP1-expressing retinal neurons (17). In any event, the assays described will set the stage to investigate the role of RPGR and mutations therein in the phenotypes here identified. Finally, LCA-linked to RPGRIP1 (and possibly XIRP3) may share some pathomechanisms to other human diseases. To this effect, the pathological relocation to the nucleus of (atypical) proteolytic fragments owing to genetic lesions in the cognate genes is known to underlie the molecular basis of several human diseases. Among others, these include some pathogenic polyglutamine proteins such as huntingtin (40–42), chimeric oncogenic proteins such as the mixed-lineage leukemia gene (43) and the genetically truncated small conductance calcium-activated K⁺ channel (SKCa3/KCNN3), which is linked to schizophrenia (44).

Altogether, these data led us to build a model outlined in Figure 9. This reflects the differential role of RPGRIP1 among retinal neurons. RPGRIP1 activity is regulated by RID and by its natural partner, RPGR. In photoreceptor
neurons expressing RPGR and RPGRIP1, the localization of these is restricted to the outer segment (ROS) compartment, where their function remains elusive but nonetheless necessary for maintaining the ROS structural integrity. While genetic lesions in RPGR and/or RPGRIP1 may directly contribute to the disorganization of the outer segments, the uncoupling of RPGR from RPGRIP1 may promote the (deleterious) production of RPGRIP1 signaling byproducts (e.g. p7, p17, etc.) by preventing the constitutive sequestration of RPGRIP1 from the ROS and hence its limited proteolysis (19). These nuclear signaling byproducts could lead to the nuclear reprogramming/deregulation of discrete genes and degeneration of photoreceptors. Conversely, RPGRIP1 is likely subjected to constitutive and limited proteolysis in inner retinal neurons, as supported by subcellular fractionation experiments from ROS-depleted retinas (19). This process may also be regulated developmentally. We postulate that constitutive production of RPGRIP1 byproducts elicits modulation of the RPGRIP1-regulated genetic network, which is required for the maintenance of the inner retinal circuitry. The pathological disturbance of the levels and production of RPGRIP1 byproducts may deregulate RPGRIP1-dependent gene expression and consequently the function of inner retinal neurons. Identification of novel molecular partners interacting with selective domains of RPGRIP1/RPGR will serve to test and to refine further the model of RPGRIP1 (and RPGR) function presented.

MATERIALS AND METHODS

Primary antibodies and reagents

Antibodies 38, and 22 and 23, respectively, against RID and SMC/CC domains of bRPGRIP1, were described elsewhere (13). The mouse monoclonal anti-HA and -EGFP antibodies were from Abcam (Cambridge, MA, USA) and BD Biosciences

Figure 9. Model of the role of RPGRIP1 in neuroretinal function and molecular pathogenesis of LCA/XIRP3. Schematic diagram of RPGRIP1 depicting the nuclear (translocation) domain (ND) identified in this work with the consensus nuclear localization signals (NLS) in human (h) and bovine (b). Normal and pathological (e.g. D1114G mutation) proteolytic sites are noted with filled and dashed arrows. The model depicts two independent roles of p175/RPGRIP1 in retinal function (for detailed information see discussion), one in photoreceptor neurons and another in inner (post-receptoral) neurons. In the photoreceptors, RPGRIP1 (p175) is sequestered in the rod outer segments and it is not subjected to proteolysis. This process may be pathogenic in photoreceptors upon genetic lesions in RPGR and/or RPGRIP1 (p175). In mature post-receptoral neurons, RPGRIP1 (p175) is under constitutive proteolysis (possibly owing to the lack of RPGR and/or other factors) leading to the production of a processed and stable nuclear factor, p7. The mutations, D1114G and ΔE1279, in RPGRIP1 (p175) have several antagonistic effects on RPGRIP1 function. Among these are the effects on the interaction with RPGR, presence of SMC/CC in the nuclear/cytosolic compartments and production of proteolytic intermediate and stable products (p17).
mAb414 and anti-calnexin antibody were from Convenance (Richmond, CA, USA) and Sterngagen (British Columbia, Canada), respectively. COS7 cells were obtained from ATCC (Rockville, MD, USA). Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa-594 and Alexa-488 were from Molecular Probes (Eugene, OR, USA). Prestained SDS–PAGE markers were from New England Biolabs (Beverly, MA, USA). The coding sequences of bRPGRP1P1, bRPGRP1P1b and mRPGRIP1b were expressed under the control of CMV promoter and subcloned in-frame with EGFP in the pExpress (26), which was engineered whenever applicable with a C-terminal HA- and Flag-tag fused to a tetracysteine motif. All constructs were verified by DNA sequencing.

Yeast two-hybrid assays

Interaction and growth assays in yeast have been described in detail elsewhere (14). The maximum specific growth speed ($\mu_{\text{max}}$) was determined by calculating $\mu_{\text{max}} = (\ln(x_t) - \ln(x_0))/t$, where $x_t$ is the OD$_{\text{600}}$ of the culture at $t = t$, $x_0$ is the OD$_{\text{600}}$ at $t = 0$ and $t$ is the time between $x_0$ and $x_t$. Assays were performed with three independent clones and three samples of each clone; the results were averaged and the standard deviations calculated. Conditions of solid growth assays under stimuli in SD-dropout agar plates without Leu, Trp and His were the following: sorbitol was used at 1.3 M, heat-shock was carried out by quickly washing attached cells with PBS followed by 42°C for 1 h every day, pH of minimal drop-out media was adjusted to 4.5 with HCl, and DMSO was 2%.

Site-directed and deletion mutagenesis and plasmid construction

Site-directed mutagenesis was carried out by PCR with one pair of complementary primers comprising single point mutations and another pair flanking upstream and downstream a given mutation. Deletion mutagenesis was carried out with pairs of primers against domains of interest, one of these comprising the mutation of interest. PCR products were subcloned into pExpress/EGFP vectors and verified by sequencing.

Transfection of COS7 cells

COS7 cells were singly transfected with EGFP-constructs (1.5 µg) using FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) as per the manufacturer’s instructions. Cells were grown to ~60–70% confluence in Dulbecco’s modified Eagle’s medium (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% CO$_2$ and 95% O$_2$. Cells were fixed (2% paraformaldehyde) and processed for immunocytochemistry. Quantitation assays were carried out 20 h after transfection.

Immunocytochemistry and microscopy

COS7 cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) and collagen-coated 35 mm glass bottom culture dishes (MatTek Corp., MA, USA) at 5% CO$_2$/37°C for ~2 days and processed for immunocytochemistry. Primary antibodies were used at concentrations ~3–5 µg/ml. Alexa 488- and Alexa 594-conjugated secondary antibodies (2.5 µg/ml) (Molecular Probes) were used for visualization of proteins. Crossover of fluorescent probes, background and autofluorescence were found to be negligible. Visualization of specimens and localization of proteins were carried out by wide-field epifluorescence microscopy on a Nikon TE2000 inverted research microscope equipped with appropriate excitation and emission filter wheels and Plan Apochromat optics (100 x, 60 x and 40 x oil objectives with NA of 1.4 objectives) and encoded motorized Z-Stage (Prior Scientific Instruments). All images were captured with a CCD camera (CoolSNAP HQ; Ropers Scientific) at identical acquisition parameters, non-saturating integration levels, 12-bit mono black/white and then pseudo-colored. Exposure times were kept constant for quantitation analysis. Images were analyzed using Metamorph Software v6.2 (Universal Imaging).

Preparation of cell homogenates, subcellular fractions and western blot analysis

Western analysis of subconfluent (60–70%) cells were carried out by quickly washing attached cells with PBS followed by cell detachment, lysis in 3 x SDS-sample buffer [5% w/v SDS, 0.15 M Tris–HCl (pH 6.7) and 30% glycerol] and several passages through a 25G5/8 needle and 1:3 dilution in RIPA buffer (25 mM Tris, pH 8.2, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate and 0.1% SDS) containing 10 mM iodoacetamide with complete protein inhibitor cocktail (Roche) as previously described (19). Nuclear and cytosolic fractions of COS7 cells were prepared in the presence of Halt protease inhibitor cocktail with NE-PER nuclear and cytoplasmic extraction reagents as per manufacturer instructions (Pierce, Rockford, IL, USA). Protein concentration was measured by the BCA method using BSA as the standard. Western blots (~125 ng/ml of antibody) were performed exactly as described previously (45). Blots were developed with a SuperSignal chemiluminescence substrate (Pierce).

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REFERENCES


