The X-linked retinitis pigmentosa protein RP2 facilitates G protein traffic

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The X-linked retinitis pigmentosa protein RP2 is a GTPase activating protein (GAP) for the small GTPase Arl3 and both proteins are implicated in the traffic of proteins to the primary cilia. Here, we show that RP2 can facilitate the traffic of the Gβ subunit of transducin (Gβ1). Glutathione S-transferase (GST)-RP2 pulled down Gβ1 from retinal lysates and the interaction was specific to Gβ1, as Gβ3 or Gβ5L did not bind RP2. RP2 did not appear to interact with the Gβ:Gγ heterodimer, in contrast Gγ1 competed with RP2 for Gβ binding. Overexpression of Gβ1 in SK-N-SH cells led to a cytoplasmic accumulation of Gβ1, while co-expression of RP2 or Gγ1 with Gβ1 restored membrane association of Gβ1. Furthermore, RP2 small interfering RNA in ARPE19 cells resulted in a reduction in Gβ1 membrane association that was rescued by Gγ1 overexpression.

The interaction of RP2 with Gβ1 required RP2 N-terminal myristylation and the co-factor C (TBCC) homology domain. The interaction was also disrupted by the pathogenic mutation R118H, which blocks Arl3 GAP activity. Interestingly, Arl3-Q71L competed with Gβ1 for RP2 binding, suggesting that Arl3-GTP binding by RP2 would release Gβ1. RP2 also stimulated the association of Gβ1 with Rab11 vesicles. Collectively, the data support a role for RP2 in facilitating the membrane association and traffic of Gβ1, potentially prior to the formation of the obligate Gβ:Gγ heterodimer. Combined with other recent evidence, this suggests that RP2 may co-operate with Arl3 and its effectors in the cilia-associated traffic of G proteins.

INTRODUCTION

Retinitis pigmentosa (RP) describes a clinically and genetically heterogeneous group of inherited retinal dystrophies, which are characterized by progressive photoreceptor cell degeneration from the peripheral to the central retina. X-linked RP (XLRP) is the most severe form of RP with mutations in the RP2 gene accounting for ~15% of all XLRP cases (1–3). RP2 is a ubiquitously expressed 350-amino acid protein, which does not appear to be enriched in retina (1,4). It is therefore currently unclear why mutations in the RP2 gene lead to a retina-specific phenotype.

Recent data have implicated RP2 in primary cilia-associated traffic (5–7). RP2 is present at the ciliary apparatus, the cilium associated centriole and the basal body of photoreceptor cells in addition to the Golgi, periciliary ridge and plasma membrane (5). Therefore, RP2 is ideally localized to couple the movement of proteins destined for cilia from the endomembrane system to the base of the cilium, and via intraflagellar transport (IFT) into the cilium. RP2 is a GTPase activating protein (GAP) for the small GTPase Arl3 (8), which is important for cilia function (9,10). Importantly, RP2 small interfering RNA (siRNA) phenocopies expression of a constitutively active form of Arl3 (Arl3-Q71L) and reduces Golgi cohesion and disrupt IFT20 localization (5). Arl3-GTP binds the effector protein UNC119 (also known as HRG4), and a ternary complex of RP2:Arl3:UNC119 may form transiently before Arl3-GTP hydrolysis is stimulated by RP2 (11). Recently, UNC119 was shown to bind the acyl chains and N-termini of specific G protein α subunits (12). UNC119 deletion...
Heterotrimeric G proteins mediate the transduction of extracellular signals into biochemical, cellular responses. Transducin is a heterotrimeric G protein, which couples phototransduction between the light-activated protein rhodopsin and the effector enzyme cGMP phosphodiesterase (PDE) in rod photoreceptors. Activation of rhodopsin by light catalyses the binding of GTP to the transducin α subunit (Go1), thereby triggering the separation of Go1 from the transducin βγ heterodimer (Gβ1:Gγ1) (reviewed in 13–15). Recently, the molecular mechanisms of G protein assembly and trafficking have started to become clear (reviewed in 16). In a current model of G protein synthesis and Gβ-Gγ heterodimer formation, the chaperonin containing TCP-1 (CCT) chaperone complex binds to nascent Gβ and promotes correct folding of Gβ. Phosducin-like protein 1 (PhLP1) acts as a co-chaperone by binding to CCT:Gβ to form a ternary complex. PhLP1 phosphorylation stimulates the release of PhLP1:Gβ from CCT, allowing binding of Gγ to form PhLP1:Gβ:Gγ. However, it is not clear where in the cell newly synthesized Gβ interacts with Gγ and whether Gβ:Gγ dimer formation proceeds or follows isoprenylation of Gγ.

In this study, we report the identification of the β1 subunit of transducin (Gβ1) as a novel RP2 interaction partner. Importantly, RP2 facilitates Gβ1 traffic suggesting a potential mechanism for enhanced photoreceptor sensitivity to mutations in RP2 as opposed to other primary cilia.

**RESULTS**

**RP2 interacts with the β1 subunit of transducin**

Mutations in RP2 cause a retina-specific phenotype, even though the protein is ubiquitously expressed. Despite the progress that has been made in recent years, the function of RP2 in retina is still unknown. The identification of retina-specific RP2 interaction partners could, therefore, provide vital clues to fully understand RP2 pathogenesis. This is especially important as the identified RP2 interacting proteins identified to date, Arl3, N-ethylmaleimide sensitive factor (NSF), polycystin 2 (PC2) and importin β2, are also ubiquitously expressed (6,7,17,18). To identify potential retina-specific RP2 interacting proteins, a GST pull-down using porcine retinal lysate was performed with full-length recombinant GST-RP2. Comparison of GST (control) with the GST-RP2 retina lysate pull-down showed several unique bands, including one at 37 kDa in the GST-RP2 lane (Supplementary Material, Fig. S1). Subsequent analysis by ESI-MS/MS revealed that a 37 kDa band contained three peptide sequences with significant (>35) MASCOT ion scores that matched the highly conserved β subunit of transducin, Gβ1 (Supplementary Material, Fig. S2). The specific pull-down of Gβ1 with GST-RP2 was confirmed by analysing a porcine retinal pull-down experiment by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent western blotting with a Gβ1 antibody (Fig. 1A).

Gβ subunit structure is highly conserved between tissues and species. To determine whether the interaction of RP2 with Gβ was specific to the Gβ1 subunit, or shared with other G protein β subunits, co-immunoprecipitation (co-IP) assays were performed in human SK-N-SH cells, which do not express detectable endogenous RP2 (data not shown). Co-expression of human FLAG-tagged constructs of Gβ1, Gβ3 or Gβ5L, all of which are expressed in retina, with RP2-GFP confirmed that RP2 specifically interacted with the β1 subunit of transducin, but not with the other Gβ subunits (Fig. 1B).

The Gβ1 subunit is part of the heterotrimeric G protein transducin (Go1:Gβ1:Gγ1) and Gβ1 forms a tightly associated, obligate heterodimer with the Gγ1 subunit. We therefore explored the alternative hypotheses that either RP2 is able to form a ternary complex with the Gβ1:Gγ1 dimer, or Gγ1 and RP2 compete for binding to Gβ1. Initially, the GST-RP2 retinal lysate pull-downs were western blotted with antibodies against Gγ1 (Supplementary Material, Fig. S3). Gγ1 was not detected in the pull-down with GST-RP2 compared with GST alone. To investigate the potential association of Gγ1 with RP2 and Gβ1 further, SK-N-SH cells were transfected with RP2-GFP and FLAG-Gβ1 with or without haemagglutinin (HA)-Gγ1, and co-IPs were performed. Expression of HA-Gγ1 with RP2 and Gβ1 reduced the recovery of RP2 and Gβ1 with each other, indicating competitive binding of RP2 and Gγ1 for Gβ1 (Fig. 1C). As expected, FLAG-Gβ1 co-precipitated with HA-Gγ1; however, no interaction was observed between HA-Gγ1 and RP2, suggesting RP2 does not form a heterotrimer with Gβ1:Gγ1 (Fig. 1C).

The specificity of the respective co-IPs was confirmed using non-targeting antibodies from the same species as the IP antibody (IgG, Fig. 1C and Supplementary Material, Fig. S4).

**RP2 enhances Gβ1 traffic to the plasma membrane**

Since RP2 did not bind the Gβ1:Gγ1 dimer, we next investigated the effect of RP2 expression on Gβ1 localization. SK-N-SH cells were transfected with FLAG-Gβ1 and RP2-GFP (or GFP) and the cell lysates were separated into membrane, cytosol, nuclear, ‘cytoskeleton’ and pellet fractions by differential sedimentation and detergent solubility (Fig. 1D). When Gβ1 was expressed with GFP, the majority of Gβ1 localized to the pellet and ‘cytoskeleton’ fraction, which correspond to the most detergent insoluble fractions. In contrast, co-expression of Gβ1 with RP2-GFP shifted Gβ1 from the pellet fraction into the membrane fraction, as shown by co-fractionation with RP2-GFP, which is membrane associated (4,19), and the endoplasmic reticulum (ER) protein calnexin (CNX). Co-transfection of FLAG-Gβ1 with HA-Gγ1 and either GFP or RP2-GFP also resulted in a relocalization of Gβ1 to the membrane fraction, independent of RP2 (Fig. 1D).

RP2 is targeted to the plasma membrane of transfected cells by N-terminal dual acylation (4,19). Interestingly, RP2-GFP co-localized with FLAG-Gβ1 (Fig. 3A), suggesting a potential enhancement of Gβ1 association with membranes and the plasma membrane in particular. To determine the effect of RP2 on Gβ1 plasma membrane targeting, SK-N-SH cells were transfected with FLAG-Gβ1 and either untagged RP2 [RP2 in pBKCMV; (4)] or empty pBKCMV (CMV) vector. Cells were stained for FLAG and cadherin, which was used as an independent plasma membrane marker. Expression of Gβ1 alone resulted in a mainly punctate, cytoplasmic staining...
pattern for Gβ1 (Fig. 1E). However, when Gβ1 was co-expressed with RP2, Gβ1 staining was observed at the plasma membrane, as indicated by co-localization with cadherin (Fig. 1E). Expression of Gβ1 with HA-Gγ1 also resulted in the translocation of Gβ1 from the cytosol to the plasma membrane, irrespective of whether RP2 was present (Fig. 1E). Co-localization was confirmed by image analyses (Supplementary Material, Table S1). Quantification of Gβ1 staining showed that Gβ1 only reached the plasma membrane in 20% of cells in the absence of RP2 or HA-Gγ1 (Fig. 1F). In contrast, significantly more cells had Gβ1 plasma membrane staining in the presence of RP2 (82%), HA-Gγ1 (67%) or RP2 and HA-Gγ1 (82%). These data suggest that the expression of RP2 and/or HA-Gγ1 can enhance the traffic of Gβ1 to membranes.

RP2 siRNA reduces Gβ1 traffic to membranes
To test the physiological relevance of the RP2:Gβ1 complex formation, human retinal epithelial cells (ARPE19), which endogenously express RP2 (5,20), were treated with siRNAs to RP2, the RP2 interacting protein Arl3 or a scrambled control. In control siRNA-treated ARPE19 cells, FLAG-Gβ1 had plasma membrane staining pattern, as shown by the overlap with cadherin in over 80% of cells (Fig. 2A and B).
Knock-down of RP2 significantly reduced the plasma membrane localization of FLAG-Gβ1 (only 22% of cells, Fig. 2B) and the protein had a punctate staining pattern in the cytoplasm (Fig. 2A). Treatment with Arl3 siRNA had no effect on FLAG-Gβ1 localization. In SK-N-SH cells, HA-Gγ1 was able to traffic Gβ1 independently of RP2. We, therefore, predicted that HA-Gγ1 would be able to restore FLAG-Gβ1 trafficking to the plasma membrane in RP2 knock-down cells. Indeed, co-expression of HA-Gγ1 with FLAG-Gβ1 in RP2 knock-down cells significantly restored FLAG-Gβ1 plasma membrane localization in over 80% of cells (Fig. 2A and B; Supplementary Material, Table S2).

ARPE19 cells express detectable levels of endogenous Gβ1 (Fig. 2C), so we investigated the effect of RP2 siRNA on endogenous Gβ1 localization using subcellular fractionation. In control siRNA-treated cells, endogenous Gβ1 was almost exclusively in the membrane fraction, as were RP2 and CNX (Fig. 2C). Strikingly, depletion of RP2 by siRNA resulted in a shift of Gβ1 from the membrane to the cytosol fraction, confirming that RP2 facilitates the membrane association of Gβ1.

Gβ1 membrane targeting requires RP2 membrane association and occurs via the Tbcc-homology domain

To elucidate the mechanisms by which RP2 causes the translocation of Gβ1 to membranes, SK-N-SH cells were transfected with wild-type and mutant variants of RP2, including acylation mutants and pathogenic point mutations (4). In the presence of GFP, FLAG-Gβ1 had a punctate cytoplasmic stain that did not overlap with the diffuse cytoplasmic and nuclear localization of GFP. In contrast, wild-type RP2-GFP (RP2-WT) was targeted to the plasma membrane and caused a shift in FLAG-Gβ1 to overlap with RP2 (Fig. 3A; Supplementary Material, Table S3). The palmitoylation RP2 mutant (C3S), which reduces RP2 plasma membrane association and enhances endomembrane association (4,19), and the patient mutation L253R, which destabilizes the structure of the C-terminal NDK-like domain (5,21), retained the ability to translocate FLAG-Gβ1 to the plasma membrane. In contrast, the plasma membrane targeting of FLAG-Gβ1 was not stimulated by the RP2 myristoylation mutant (G2A), which blocks RP2 acylation and membrane association (4,19), or the patient mutation R118H, which inhibits RP2 GAP activity for Arl3 (8) (Fig. 3A and B). These findings were confirmed using a sub-cellular fractionation assay. SK-N-SH cells were co-transfected with FLAG-Gβ1 and either wild-type or mutant RP2. Control wild-type RP2 (RP2-WT) with FLAG-Gβ1 resulted in both proteins partitioning to the membrane fraction (Fig. 3C). As previously reported (4,19), the RP2 myristoylation mutant G2A did not fractionate to membranes and the majority of the RP2-G2A protein was retained in the cytosol and nuclear fractions. RP2-G2A did not shift FLAG-Gβ1 to the membrane fraction from the cytoskeleton or pellet fraction (Fig. 3C). In contrast, the RP2 palmitoylation mutant (RP2-C3S) was present in the membrane fraction and could enhance FLAG-Gβ1 recruitment to membranes, albeit...
to a lesser extent than RP2-WT. This indicates either a slight reduction in trafficking of Gβ1 to membranes by RP2-C3S or that the loss of one lipid modification might reduce the stability of the RP2-C3S and Gβ1 membrane association. The fractionation of RP2-L253R resembled that of wild-type RP2 and the mutation did not inhibit the re-distribution of FLAG-Gβ1. However, following co-expression with RP2-R118H, FLAG-Gβ1 membrane association was lost, even though this RP2 mutant was still present in the membrane fraction (Fig. 3C).

Figure 3. Gβ1 membrane targeting requires RP2 membrane association and the TBCC domain. (A) Myristoylation of RP2 is required for Gβ1 membrane targeting. Immunofluorescence showing the localization of overexpressed FLAG-Gβ1 (red) in SK-N-SH cells with GFP (green), RP2-GFP (green) or RP2-GFP mutants (green), as indicated. Note overlap of RP2-GFP, RP2-C3S and RP2-L253R fluorescence with FLAG-Gβ1 signal. The inset shows a higher magnification of the region boxed in the merge image, scale bar 10 μm. (B) Quantitation of Gβ1 plasma membrane association showing significant reduction in plasma membrane localization with the RP2 myristoylation mutant (G2A) and the RP2 pathogenic mutation in the TBCC domain (R118H). Cells were scored for overlap of the Gβ1 signal with cadherin at the plasma membrane (PM) as a percentage of all transfected cells (n = 3), values are means ± SEM; ***P < 0.001. (C) RP2 myristoylation and R118 are required for membrane association of Gβ1. Subcellular fractionation of SK-N-SH cells co-transfected with FLAG-Gβ1 and either wild-type or mutant RP2-GFP as indicated. Presence of RP2 and Gβ1 in fractions was determined by western blotting. FLAG-Gβ1 co-transfected WT-RP2 resulted in membrane association of both proteins (M; panel 2). RP2-G2A myristoylation mutant (panel 3) did not associate with membranes or stimulate translocation of Gβ1 from the cytoskeleton (CS) or pellet (P) fractions. RP2 palmitoylation mutant (RP2-C3S) was present in the membrane fraction and enhanced FLAG-Gβ1 recruitment to this fraction (M; panel 4), but to a lesser extent than RP2-WT (panel 2). Panel 5 shows FLAG-Gβ1 membrane association was lost even though RP2-R118H was present on membranes (M). RP2-L253R (panel 6) had no effect on Gβ1 localization and was comparable to WT-RP2 (panel 2). Total (T); membrane fraction (M); cytosol (C); cytoskeleton (CS); nuclear (N); pellet (P). (D) Pull-down of Gβ1 with domains and mutants of RP2. SK-N-SH cell lysates overexpressing FLAG-Gβ1 were used in pull-down assays with His-tagged RP2 fragments or point mutations as indicated. Upper panel shows that the TBCC domain of RP2 (RP2-41-250) pulls down Gβ1, whereas an RP2 C-terminal fragment (RP2-180-350) does not interact. Binding of Gβ1 to the TBCC domain is abolished with the RP2-R118H pathogenic mutant. A pathogenic mutation in the C-terminal domain (RP2-L253R) does not affect binding. Lower panel shows equal loading of RP2 proteins.
To determine which domain mediates the interaction between RP2 and FLAG-Gβ1, pull-down experiments using recombinant His-tagged RP2 constructs were performed. SK-N-SH cells were transfected with FLAG-Gβ1 and cell lysates were incubated with either full-length His-RP2-WT, His-RP2-R118H, His-RP2-L253R, an N-terminal truncation His-RP2-41-350 or the C-terminal fragment His-RP2-180-350 as bait. FLAG-Gβ1 was successfully pulled down using the RP2-WT, RP2-L253R mutant and RP2-41-350 domain construct, but was unable to bind to RP2-R118H or RP2-180-350 (Fig. 3D). These results demonstrate that the co-factor C (TBCC) homology domain of RP2 is required for binding Gβ1, while RP2 membrane association through myristoylation is essential for trafficking Gβ1 to membranes. In contrast, palmitoylation, or the C-terminal domain, was not required for Gβ1 binding and/or trafficking to membranes.

The effect of Arl3 on RP2 traffic of Gβ1

RP2 binds preferentially to Arl3-GTP (5,17,21) and is a negative regulator (GAP) for Arl3 (8). Because the RP2 TBCC domain, which binds Arl3, and the GAP critical arginine finger residue R118 appear to be important for Gβ1 binding, we tested the hypothesis that Arl3 activation would affect the RP2:Gβ1 interaction. First, we established whether RP2, Arl3 and Gβ1 can form a ternary complex, or if a constitutively active Arl3-GTP conformational mimic Arl3-Q71L would compete with Gβ1 for binding to RP2. IP of RP2 confirmed the known preference of RP2 for binding Arl3-Q71L over Arl3-T31N, and revealed a reduced interaction between RP2 and Gβ1 in the presence of Arl3-Q71L compared with Arl3-T31N (Arl3-GDP) or no Arl3 overexpression (Fig. 4A). IP of FLAG-Gβ1 confirmed the reduced recovery of RP2 with Gβ1 in the presence of Arl3-Q71L and no interaction was detected between Arl3 and Gβ1 (Fig. 4A). Furthermore, co-expression of Arl3-Q71L with RP2-GFP and FLAG-Gβ1 dramatically altered the localization of Gβ1 (Fig. 4B). Arl3-Q71L caused a shift in Gβ1 localization from the plasma membrane to the cytosol (Fig. 4B and C). In contrast, Arl3-T31N did not alter the RP2-mediated re-localization of Gβ1 (Fig. 4B and C; Supplementary Material, Table S4). This was confirmed using subcellular fractionation in ARPE19 cells, in which FLAG-Gβ1 is normally membrane associated (Figs 2 and 4D). Expression of Arl3-WT or Arl3-T31N did not affect FLAG-Gβ1 membrane association, whereas Arl3-Q71L led to a reduction in the amount of FLAG-Gβ1 that was present in the membrane fraction (Fig. 4D). Arl3 was present mainly in the cytosol fraction, with Arl3-Q71L enriched in the membrane fraction in the presence of RP2, as has been reported previously (5). Collectively, these data indicate that Arl3-Q71L and Gβ1 compete for binding to RP2 and suggest that Arl3-GTP can displace Gβ1 from an RP2:Gβ1 complex.

RP2 facilitates the loading of Gβ1 to Rab11 vesicles

The mechanisms of transducin traffic to the photoreceptor outer segment are still unclear. Since rhodopsin transport vesicles are enriched with Rab11 (22), we investigated the potential co-localization of RP2 and/or Gβ1 with Rab11. Interestingly, expression of DsRed-Rab11 with RP2-GFP in SK-N-SH cells led to the recruitment of RP2 to the DsRed-Rab11 vesicles (Fig. 4E; Supplementary Material, Table S4). Similarly, when DsRed-Rab11 was expressed in ARPE19 cells, this resulted in the recruitment of endogenous Gβ1 (Fig. 4E) to Rab11 vesicles. To test if the recruitment of Gβ1 to these vesicles was dependent on RP2, we co-expressed DsRed-Rab11 with FLAG-Gβ1 in the presence or absence of RP2 in SK-N-SH cells (Fig. 4F). FLAG-Gβ1 was only recruited to Rab11 vesicles in the presence of RP2, suggesting that RP2 facilitates the traffic of FLAG-Gβ1 via Rab11 vesicles.

DISCUSSION

Here we report the identification of the transducin beta subunit (Gβ1) as a novel interaction partner of RP2. Previously, RP2 has been shown to interact with Arl3 (17), NSF (18), PC2 (6), and importin β2 (7). Therefore, Gβ1 is the first RP2 interactor that has a specific role in photoreceptor function thus providing a functional link with the retinal specificity arising from mutations in RP2. Gβ1 is widely expressed, but together with other transducin subunits plays an essential role in phototransduction. Interestingly, the interaction was specific for Gβ1, as RP2 did not co-immunoprecipitate with Gβ3 or Gβ5L. Gβ3 is closely related to Gβ1 (84% identity, 92% similarity), whereas Gβ5L is more divergent (53% identity). Gβ1 and Gβ3 differ most at their N-termini, which forms a coiled coil interaction with Gγ. Interestingly, we show that RP2 and Gγ1 appear to compete for binding to Gβ1 supporting the hypothesis that they may be competing for the same binding site at this divergent N-terminus. This hypothesis will require empirical determination using chimeric proteins and/or site-directed mutagenesis of Gβ subunits.

The biogenesis and assembly of heterotrimeric G protein subunits is complex and requires the action of several molecular chaperones and post-translational modifications (reviewed in 16). For example, nascent chains of the Gβ1 subunit, like other seven blade β-propeller WD40 proteins, are encapsulated in the cytosolic chaperonin CCT, to facilitate folding and prevent aggregation (23–25). The CCT co-chaperone PhLP1 facilitates the release of Gβ and assembly of the Gβ:Gγ heterodimer (26–29). In parallel, Gγ synthesis is facilitated by another chaperone DRiP78 (30) prior to membrane association via prenylation (16), yet the site of heterodimer formation is still not clear. Intriguingly, there are several parallels between Gβ:Gγ assembly with the formation of the tubulin α:β heterodimer and the role of the RP2 homologue TBCC (31,32). Similar to Gβ, tubulin subunits require CCT to fold correctly, whereas upon release from CCT the assembly of the tubulin heterodimer is facilitated by tubulin folding cofactors A–E. TBCC acts with TBCD and TBCE to facilitate the formation of the tubulin heterodimer and stimulates GTP hydrolysis by the tubulin β subunit within the heterodimer. The data presented here support an analogous role for RP2 in binding Gβ1 prior to formation Gβ:Gγ heterodimer (Supplementary Material, Fig. S5).

Overexpression of Gβ1 led to the formation of detergent insoluble Gβ1 and a failure to associate with membranes.
This insolubility was suppressed by either RP2 expression or overexpression of Gγ1, and both stimulated Gβ1 membrane association. When RP2 was reduced by siRNA, overexpression of Gγ1 restored membrane association. This suggests that RP2 is not essential for Gβ1 biogenesis and assembly but rather acts to facilitate the assembly of the Gβ1:Gγ1 heterodimer by sequestering Gβ1, preventing its aggregation and positioning it close to the membrane prior to formation of the stable heterodimer with Gγ1. Gγ1 is required to maintain the level of both Ga1 and Gβ1 in photoreceptors (33); therefore, RP2 does not appear to be able to replace Gγ1 in stabilizing Gβ1 or forming the heterotrimeric complex with Ga1. Gγ1−/− mice display severe retinal degeneration, which cannot be rescued by knock-out of Ga1, indicating that rod photoreceptor death is not a result of abnormal Ga1 signalling. It has been proposed that rods degenerate because of the stress imposed by the over-production of free, unbound Gβ1, which is unable to form a functional dimer with Gγ1 (33). Therefore, RP2 could have a role in the photoreceptor machinery to reduce the stress caused by free Gβ1, by sequestering free Gβ1.
Gβ1 and facilitating the assembly and membrane association of the Gβ1:Gγ1 heterodimer.

The Gβ1:Gγ1 heterodimer must be assembled prior to association with Gα (16). Gα1 is modified by N-terminal acylation that facilitates its membrane association independently of Gβ1:Gγ1. The Arl3 effector UNC119 binds the acylated N-terminus of Gα1 and facilitates the traffic of the Gα1 subunit (12). The interaction of UNC119 with Gα1 has been independently confirmed (34). However, it is currently not clear if UNC119 binds GTP-Gα1 after dissociation from Gβ1:Gγ1 (12), or if UNC119 binding stimulates dissociation of the heterotrimer (34), indicating more of an inhibitory role in G protein signalling. Normally, in the dark, Gα1 is concentrated in the outer segment and traffics to the inner segment upon bright light illumination, by contrast in Unc119−/− mouse retina Gα1 is present in the inner segment in the dark and shows a failure to traffic to the outer segment on dark adaptation (12). Similarly, in C. elegans unc-119 mutants, the Gα proteins ODR-3 and GPR-13 do not traffic correctly in ciliated neurons (12). Interestingly, the related prenyl-binding protein and Arl2/3 effector PDEδ (also known as PrBP) is required for correct traffic of Gβ1:Gγ1, as demonstrated in the Pdeδ−/− mouse retina where Gγ1 accumulates in the inner segment (35). Therefore, it is interesting to speculate that the RP2 may act with UNC119 and/or PDEδ to facilitate G protein traffic.

Recently, it was discovered that Arl2-GTP and Arl3-GTP stimulate the release of prenylated proteins from PDEδ (36). The effect of Arl3 on UNC119:Gα binding is unknown, but it is likely that Arl3-GTP will also lead to release of the acyl chain from the UNC119 and allow membrane association of Gα1. Interestingly, Arl3-Q71L competes with Gβ1 for RP2 binding, suggesting that RP2 binding to Arl3-GTP would also stimulate release of Gβ1 from RP2. Therefore, Arl3-GTP stimulates the release of Gγ1 from PDEδ (or Gα1 from UNC119) at the same subcellular locale that Gβ1 is released by RP2. This would enable heterodimer (or heterotrimer) assembly at a specified membrane surface, possibly at the ER, Golgi or a transport vesicle, thereby stimulating traffic (Supplementary Material, Fig. S5). Furthermore, RP2 can form a ternary complex with Arl3 and UNC119 in the presence of a non-hydrolysable GTP analogue (11). The GAP activity of RP2 on Arl3 causes the release of effectors, such as UNC119, from Arl3, as effectors have a low affinity for Arl3-GDP. Therefore, the action of RP2 on Arl3-GTP would release UNC119 for further cycles of Gα1 acyl chain binding.

Correct trafficking of proteins to the outer segment is not only vital for phototransduction, but also for photoreceptor survival. For example, mislocalization of even a small fraction of rhodopsin may have a detrimental effect on photoreceptor cell function (37). Cargo is sorted at the Golgi and following budding, vesicles are delivered to their target membranes in a multistep process, which requires transport, tethering and fusion. A key player in targeting vesicles to their fusion sites is the exocyst, a multiprotein complex that mediates the delivery of vesicles. The exocyst is a downstream effector of the GTPases Rab8 and Rab11 (38), which in concert with ARF4 regulate post-Golgi budding and motility of rhodopsin-containing vesicles (22,39). These GTPases function in concert with SNARE proteins in photoreceptor outer segment membrane trafficking and fusion as part of the outer segment renewal process (40). RP2 interacts with NSF, an ATPase that co-operates with SNAREs in membrane fusion (18). RP2 and NSF co-localize at the base of the outer segment (18), suggesting a role for RP2 in regulation of SNARE-mediated protein delivery to the ciliary base or periciliary ridge. Here, we show that RP2 is recruited to Rab11-positive vesicles and facilitates the association of Gβ1 with Rab11-positive vesicles. Interestingly, UNC119 regulates the traffic of the tyrosine kinase Lck via Rab11 endosomes in immunological synapse formation (41). Therefore, we suggest that RP2 acts with Arl3 effectors to target the assembly and traffic of specific cargo proteins to Rab11 vesicles and thereby to cilia.

The precise function of RP2 in G protein traffic remains to be defined, but collectively our data could support a role for RP2 in facilitating Gβ1:Gγ1 heterodimer assembly and membrane targeting orchestrated by Arl3 in co-operation with Arl3 effectors. Whether this effect on traffic extends to influencing light/dark adaptation-associated traffic events, as suggested for UNC119 (12,34), will require the development and characterization of RP2 knock-out animal models. To date, zebrafish models have been developed (6,42), but these models show abnormal retinal development and non-retinal phenotypes, so it has not been possible to study RP2 knock-out on photoreceptor function. The development of other models will be important to help determine the role of RP2 in cilia-associated traffic and photoreceptor function.

MATERIALS AND METHODS

Cell culture and cDNA constructs

Human retina pigment epithelial (ARPE19) and neuroblastoma (SK-N-SH) cells were grown in Dulbecco’s modified Eagle’s medium/F12 (Invitrogen, Paisley, UK). Serum-rich medium contained 10% (v/v) fetal bovine serum. For immunofluorescence staining, cells were cultured in eight-well chamber slides (VWR, Lutterworth, UK). Cells for western blotting were plated into six-well plates (Nunc, Fisher Scientific, UK). Transfections of plasmids were performed using Lipofectamine and Lipofectamine Plus reagent according to the manufacturer’s instructions (Invitrogen). Generation of RP2 and Arl3 mutants has been described elsewhere (4,5). pcDNA3.1 human FLAG-Gβ1, FLAG-Gβ3, FLAG-Gβ5L and the pcDNA3.1 HA-Gγ1 constructs were obtained from Missouri S&T cDNA resource centre (Missouri, USA). To create N-terminal GST-fusion constructs, full-length human RP2 was cloned into the pGEX-2T vector (GE Healthcare, Chalfont St Giles, UK). N-terminally histidine-tagged recombinant proteins HIS-RP2, HIS-RP2-R118H, HIS-RP2-L253R, HIS-RP2-41-350 and HIS-RP2-182-350 were cloned into the pTrcHisA (Invitrogen).

Antibodies

Production and characterization of affinity-purified sheep polyclonal RP2 antisera S974 has been described previously (4,19). Mouse anti-M2 FLAG, mouse anti-myc and mouse anti-HA (all 1:1000, Sigma-Aldrich, Dorset, UK) were used
to detect FLAG-Gβ1, myc-Arl3 and HA-Gγ1 constructs, respectively. Endogenous Gβ1 was detected using the C-16:sc-379 rabbit anti-Gβ1 antibody and endogenous Gγ1 with P-19:sc-373 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The rabbit anti-Arl3 antibody was a gift from Dr N. J. Cowan (New York University, USA). Rabbit anti-CN X antibody (1:500, StressGen, Gentaur, Brussels, Belgium) and pan-cadherin (1:500, Abcam) were used to stain membrane structures by western blotting or immunocytochemistry.

### Purification of recombinant proteins

GST, GST-RP2, His-tagged wild-type RP2, RP2-R118H, RP2-L253R or the RP2 deletion constructs 41–350 and 180–350 were expressed in OneShot TOP10 cells (Invitrogen). Protein expression was induced for 2–4 h by adding isopropyl-beta-D-thiogalactopyranoside (Sigma) to a final concentration of 1 mM. GST and GST-RP2 were immobilized on glutathione sepharose beads (GE Healthcare), while His-tagged RP2 constructs were purified by immobilized metal affinity chromatography using a TALON resin (Clontech), both according to the manufacturer’s instructions.

### Retinal lysate preparation and GST pull-down

Retinai were dissected from porcine eyes (Cheal Meats, Brentwood, Essex, UK), washed in cold phosphate buffered saline (PBS) and subsequently lysed for 15 min on ice in lysis buffer [25 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), Protease inhibitor cocktail (PIC; Sigma), phosphatase inhibitor cocktail (PhlC; Sigma)]. Lysates were homogenized in a cold Dounce homogenizer and centrifuged at 16,200 g for 15 min at 4°C. The supernatant was collected and incubated over night with slow rotation at 4°C with sepharose beads coupled to either GST or GST-RP2. The beads were washed four times in lysis buffer and analysed on a ProteanII xi Cell SDS–PAGE with subsequent mass-spectrometry compatible silver stain. Briefly, the gel slab was fixed in 50% methanol with 5% acetic acid, followed by washes in 50% methanol and water. The gel was sensitized by incubation in 0.02% sodium thiosulfate for 1 min, washed in water and submerged in 0.1% silver nitrate solution for 20 min at 4°C. After several washes, the gel was developed in 0.04% formalin in 2% sodium carbonate. Development was terminated by washing the gel with 5% acetic acid.

### Mass spectrometry

Unique bands in the GST-RP2 lane, compared with the GST lane, were excised and digested in the gel, using a robotic system as described previously (43). Samples were analysed using electrospray ionization tandem mass spectrometry (ESI-MS/MS) and tandem mass spectra were recorded using a Waters Q-ToF mass spectrometer (Waters, Manchester, UK). Data obtained from ESI-MS/MS were further analysed using the MASCOT programme and proteins were considered identified when they were represented by at least two unique peptide sequences with a MASCOT ion score >35, or at least one peptide sequence with a MASCOT ion score >70.

### Immunocytochemistry (ICC)

For immunocytochemistry (ICC), cells were washed twice in PBS and either fixed in 100% ice-cold methanol for 2 min or 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and subsequently incubated for 1 h in blocking buffer [3% bovine serum albumin (BSA), 10% normal donkey serum in PBS] to avoid non-specific antibody binding. Following block, cells were incubated for 1 h with primary antibodies at the appropriate titre. After washing with PBS, cells were then incubated with fluorescent labelled (Cyanine2 or Cyanine3) secondary antibodies (1:100, Streatech, Newmarket, Suffolk, UK) for 1 h in 3% BSA in PBS. Following several washes with PBS, cells were incubated for 5 min with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS to stain the nuclei. Confocal images were obtained using the LSM510 microscope (Carl Zeiss MicroImaging) and analysed using the LSM Image Browser software (Carl Zeiss MicroImaging), prior to export and image processing and annotation using Adobe Photoshop and Illustrator. Co-localization of proteins at the plasma membrane was determined by morphological assessment of fluoroscetently labelled cells using a Nikon Eclipse 80i microscope and Nikon NIS-Elements software (Basic Research, Version 2.2, Nikon). Proteins were considered to co-localize when both red and green channel overlapped in the regions of interest (i.e. the plasma membrane) and the resulting merged image appeared yellow. Experiments were repeated three times and a minimum of 100 cells per treatment were analysed. In addition, the Pearson’s correlation co-efficient and the Mander’s co-localization co-efficient were determined to corroborate the morphological assessment and quantify co-localization, as previously described (44). Briefly, per treatment a minimum of three representative confocal images were analysed using the ImageJ JACob plug-in software (Supplementary Material, Tables S1–S4).

### RNA interference

For RNA-interference studies, ARPE19 cells, a pool of four siRNAs for RP2 and Arl3 in addition to a non-targeting control siRNA, were obtained from Dharmacon (ON-TARGET plus siRNA reagents, Chicago, IL, USA) as previously described (5).

### Co-immunoprecipitations

Co-IPs were performed using magnetic Dynabeads Protein G (Invitrogen) according to the manufacturer’s instructions. Briefly, SK-N-SH cells were plated into six-well plates and transfected as described above. Cells were lysed in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.25% deoxycholate, 1% NP-40, 1 mM EDTA, PIC, PhlC) and lysates were subsequently incubated with 25 μl of Dynabeads and primary antibodies for 1 h at room temperature on a rotating wheel. The beads were washed several times with lysis buffer before elution with 2 × SDS loading buffer and analysis by western blotting.
Subcellular fractionation assay and western blotting

To separate cytosolic, nuclear, membrane and ‘cytoskeletal’ protein fractions, SK-N-SH or ARPE19 cells were plated into six-well plates and transfected as described above. Cells were trypsinized and pellets resuspended in buffer C (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 2 mM MgCl₂, 100 mM KCl, 5 mM EDTA, 250 mM sucrose, 10% glycerol) and incubated at 4°C for 20 min with slow rotation. The cell lysate was passed through a syringe with a 27 gauge needle 60 times and a total lysate fraction was removed for sample analysis. The remaining lysate was centrifuged at 15 000 g for 10 min at 4°C. The supernatant was collected and represented the cytosolic fraction. The pellet was washed once in buffer C and subsequently resuspended in buffer N (20 mM HEPES, pH 7.9, 2 mM MgCl₂, 50 mM NaCl, 5 mM EDTA, 5% glycerol) as above. Following incubation, the lysate was centrifuged at 15 000 g and the supernatant representing the nuclear fraction was collected. The pellet was resuspended in buffer M (20 mM HEPES, pH 7.9, 2 mM MgCl₂, 100 mM KCl, 5 mM EDTA, 250 mM sucrose, 5% glycerol, 0.5% sodium deoxycholate, 1% NP-40) and the membrane fraction was collected, before a final incubation and centrifugation with buffer CS (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 2 mM MgCl₂, 150 mM NaCl, 5 mM EDTA, 250 mM sucrose, 1% SDS) to obtain the ‘cytoskeleton’ fraction. The remaining pellet was resuspended in 2× SDS loading buffer and represented the insoluble ‘pellet’ fraction. Proteins from all fractions, except the pellet, were precipitated with 5% TCA by incubation on ice for 10 min, followed by centrifugation at 15 000 g for 10 min. Pellets were washed in 70% ethanol and resuspended in 2× SDS loading buffer. For western blotting of RP2, myc-Arl3, FLAG-Gβ1 or HA-Gγ1 protein expression, crude sheep anti-RP2 antibody (1:2000), rabbit anti-Arl3 antibody (1:12000) or mouse anti-myc antibody (1:1000), mouse anti-FLAG antibody (1:1000) or mouse anti-HA antibody (1:1000) were used, respectively. Additionally, lysates were blotted with a rabbit anti-CNX antibody (1:4000). Secondary antibodies used were horseradish peroxidase conjugated goat anti-sheep, donkey anti-rabbit or goat anti-mouse antibodies (Stratech). Blots were developed using the enhanced chemiluminescence western blotting detection system (GE Healthcare).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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