C2 Domains as Protein-Protein Interaction Modules in the Ciliary Transition Zone

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INTRODUCTION

Cilia are microtubule-based organelles that project from the surface of most eukaryotic cells. Motile cilia usually have a 9 + 2 microtubule doublet structure and control fluid or particle flows over epithelial surfaces (e.g., in the airways and the fallopian tube). Primary cilia generally lack the central microtubule doublet and consist of a 9 + 0 structure. They partake in several signal transduction pathways, such as the Sonic Hedgehog and Wnt pathways, and play a role in chemosensation, mechanosensation, and thermosensation. Ciliary defects lead to pleiotropic disorders, often characterized by photoreceptor degradation, kidney cysts, altered embryonic patterning, and mental retardation. These diseases are now commonly referred to as ciliopathies (Fliegauf et al., 2007; Gerdes et al., 2009).

The ciliary axoneme grows out of the basal body and is covered by the ciliary membrane. The region most proximal to the basal body is called the transition zone and is thought to act as gatekeeper structure that controls the entry and exit of proteins into and out of the cilium. Transition fibers linking the outer doublets to the membrane and the Y-linkers in the transition zone form a physical barrier that limits the free diffusion of soluble proteins into the cilium. Active or facilitated transport processes probably are particularly relevant for transmembrane proteins, as several ciliary targeting sequences (CTSs) have been identified (e.g., in rhodopsin) (Deretic et al., 1998).

The outer segment of photoreceptors represents a highly specialized form of a primary cilium and is responsible for phototransduction. Since the cell’s metabolic machinery is present in the inner segment, the proteins required for light sensing and outer-segment maintenance are trafficked from the inner to the outer segment via the connecting cilium, which is equivalent to the transition zone in other primary cilia. Retinal degeneration as a consequence of cilia malfunction occurs in many ciliopathies, and mutations in a large number of genes are responsible for retinal degeneration.

Two proteins encoded by such genes are retinitis pigmentosa G-protein regulator (RPGR) and its molecular partner, RPGR-interacting protein 1 (RPGRIP1). Defects in the RPGR gene are a major cause of X-linked retinitis pigmentosa (RP), whereas defects in RPGRIP1 lead to the more severe Leber congenital amaurosis (LCA) syndrome (Dryja et al., 2001; Meindl et al., 1996). LCA is characterized by retinal dystrophy from birth or early childhood. Recently, RPGRIP1 has also been implicated in various forms of glaucoma (Fernández-Martínez et al., 2011). RPGR has two major isoforms: RPGR1-19 and RPGRCRF15. RPGR1-19 is produced from exon 1-19 of the X-linked retinitis pigmentosa 3 gene, whereas RPGRCRF15 contains exon 1-14 supplemented with the purine-rich intron 15 (Meindl et al., 1996; Vervoort et al., 2000). Hence, both proteins contain the same N terminus, but differ in their C terminus. The N terminus comprises a domain homologous to regulator of chromosome condensation 1 (RCC1), an exchange factor for the small G protein Ran. This RCC1 domain interacts with several proteins, such as RPGRIP1 and the phosphodiesterase 6 delta subunit (PDEδi) (Boylan and Wright, 2000; Linari et al., 1999). RPGRIP1 is a large multidomain protein consisting of an N-terminal coiled coil domain, two C2 domains (C2N and C2C), and a C-terminal RPGR-interacting domain (RID). A homolog of RPGRIP1 called the RPGRIP1-like (RPGRIP1L) protein (also called NPHP8), which possesses the same domain architecture, has been identified as well.
Several mutations have been detected in the RPGR RCC1 domain in RP patients. In RPGRIP1 and RPGRIP1L, patient mutations are present in the coiled coils, the C2 domains, and the RID. Both RPGRIP1 and RPGRIP1L interact with the ciliary transition zone protein nephrocystin 4 (NPHP4) via their C2C domain, and some of the patient mutations have been shown to disrupt this interaction (Arts et al., 2007; Roepman et al., 2005).

The expression of RPGRIP1 seems to be limited to the retina, where it is expressed in photoreceptors, in the inner retina, and in amacrine cells (Castagnet et al., 2003; Mavlyutov et al., 2002). RPGR1 and RPGRIP1L are found in other tissues as well, such as the brain and kidney (Arts et al., 2007; Hong et al., 2000). RPGR and RPGRIP1 colocalize in the outer segments of human and bovine photoreceptors (Mavlyutov et al., 2002). In mice, however, they have been found to colocalize in the connecting cilium. RPGRIP1, in the present paper we structurally and biochemically analyze the complex between the RCC1 domain of RPGR and the RID of RPGRIP1 and RPGRIP1L.

RESULTS

Structure Solution

The C-terminal domain of RPGRIP1 was previously described as an interaction partner of the RCC1 domain of RPGR. Since the exact boundaries of the RID of RPGRIP1 were not known, we created several constructs (Figure 1A). For the RCC1 domain of RPGR, we chose to work with a construct comprising amino acids 1–392 plus a C-terminal StrepII tag (hereafter referred to as RPGR).

To find the best polypeptide for the RID of RPGRIP1, we produced six constructs starting from amino acids S1047, S1065, S1084, S1091, I1102, and D1114, respectively, and ending with the C-terminal residue S1286, producing proteins of 240, 222, 203, 196, 185, and 173 residues. We refer to these proteins as RID240, RID222, RID203, RID196, RID185, and RID173, respectively. Pull-downs with Strep-tagged RPGR show that the RID constructs starting from amino acid S1047, S1065, S1084, and S1091 bind to RPGR, whereas the smaller ones starting from residues I1102 and D1114 do not (Figure 1B). To gain more insights into this interaction, we solved the crystal structure of the RPGR-RID196 complex. The best crystals diffracted to 1.83 Å and allowed us to solve the crystal structure by combining the phases obtained from a SeMet derivative of RID196 and a molecular replacement template of RPGR (Table S1). The asymmetric unit contained one copy of each molecule. Residues 7–368 of RPGR and residues 1114–1281 of RID196 were clearly visible. An unconnected, strong difference in electron density was observed at the side of the RPGR β propeller and could be unambiguously assigned to residues D1097–M1106 of the RID196 N terminus because of the anomalous signal of the residue M1106.

The Components

As described previously (Wätzlich et al., 2013), RPGR forms a seven-bladed β propeller. The root-mean-square deviation (rmsd; for Cα) between the crystal structures of RPGR alone (Protein Data Bank [PDB] ID 4JHN, chain A) and in complex with RID196 is 0.42 Å. The positions of the β sheets do not differ significantly, but there are some small variations in the connecting loop regions. The largest change is found in the long loop connecting blade 1 and blade 7 of the propeller, which forms part of the RPGR-RPGRIP1 interface. The rmsd (for Cα) between the structures of RPGR in complex with RID196 and in complex with PDEβ (PDB ID 4JHP) is 0.56 Å and shows similar differences in the loop between blades 1 and 7.

RPGRIP1 contains two sequences that have been recognized as C2 domains, designated C2N and C2C (Figure 1A). The fold of the RID was unknown, although one paper suggested it might be a C2 domain as well (Zhang and Aravind, 2012). The structure shows that the RID of RPGRIP1 displays a proper C2 domain fold (Figure S1A). It consists of an eight-stranded antiparallel β sandwich. Based on the topology of the β strands, C2 domains are classified as either type I or type II. The type II topology displays a circular permutation of the order of the β strands found in type I C2 domains. These topologies were originally described in synaptotagmin C2A (type I) and in the C2 domain of PLCζ1 (type II) (Essen et al., 1996; Sutton et al., 1995). As shown in
RID196 exhibits a type II topology. Sheet 1 comprises strands 1, 4, 7, and 8. Sheet 2 consists of strands 2, 3, 5, and 6, although the 3 strand is remarkably shorter than the other strands. A small α-helical turn is localized in the loop between β1 and β2. At the tip of the molecule where the N and C termini converge, three α helices are present in the loops connecting β4 and β5 (α2), β6 and β7 (α3), and C-terminal to β8 (α4).

The RPGR-RID196 Complex

In the crystal structure, two different interaction surfaces of RPGR and RID196 are found between symmetry-related molecules (Figure 2). Given that gel-filtration analysis shows that the two proteins form an equimolar complex (Figure S1C), we decided to use mutagenesis to identify the correct interface. In contact area 1, the loop connecting β strands 4 and 5 of RID196 touches the top of the RPGR β propeller (Figures 2A and 2B). Hydrogen bonds (H bonds) and salt bridges that formed between residues of RID196 and RPGR in this orientation are shown schematically in Figure 2F. This interface buries 878 Å². The second interface (area 2) involves an interaction between RPGR and RID196, which buries 1,401 Å² of surface (Figures 2C–2E). Furthermore, in this orientation the small N-terminal peptide of the same RID196 molecule can contact the side of the RPGR β propeller (Figure 2E) and supplies an additional surface area of 1161 Å². The latter interactions between the proteins are depicted in Figure 2G.

To assess which of these two orientations reflects the real interaction, we tested a number of mutant RID proteins in pull-downs. Since the slightly longer construct RID222 performs better in pull-downs than RID196, we chose to use this construct in all biochemical experiments. In the area 1 mutant, residues K1147, K1220, K1221, and E1222 were simultaneously replaced by alanines. In the area 2 mutant, E1121, H1174, and E1245 were substituted by alanine residues. The results show that even the quadruple area 1 mutant is still capable of interacting with RPGR, whereas only a very weak interaction is observed for the triple mutant of area 2 (Figure 3A). Circular dichroism spectroscopy measurements show that the far-UV spectra of both mutant proteins are similar to the spectrum of the wild-type (data not shown); hence, the lack of interaction of the area 2 mutant is not caused by improper folding of the protein. Consequently, we conclude that contact area 2 represents the biological relevant interface between RPGR and RID196 (Figures 2C–2E and 2G).

Thus, RID196 contacts RPGR in three regions: a central one involving interaction between a β-sheet of RID196 and some side chains (e.g., E1245, E1121, and H1174) with the large loop of RPGR containing R323, N333, and N336; a mainly hydrophobic interaction site with the RPGR loop containing F271, F279, and F281; and a third one formed by the N-terminal peptide of RID196.

To investigate this interaction in more detail, we prepared several charge-reversal mutants and evaluated their binding capacities by pull-downs (Figure 3B). In RID222, the E1245K mutation does not have an effect on RPGR binding. The E1121K mutation weakens the interaction, whereas H1174D totally abolishes binding. In RPGR, the D321K mutation does not affect the interaction with RID222, whereas R323E is no longer capable of binding to RID222. This indicates that H1174 and R323 are the most important residues for stabilizing the RPGR-RID interaction. The side chains of H1174 and R323 are orientated in a parallel fashion toward each other (Figure 2D) and the distance between them is about 3.6–3.8 Å, which is optimal for the formation of like-charged stacked interaction pairs (Heyda et al., 2010).

LCA and RP Patient Mutations

A large number of RP patient mutations throughout the RCC1 domain of RPGR have been reported. Judging from the RPGR crystal structure, some of these mutations are considered to
destabilize the β-propeller domain (Wätzlich et al., 2013). Some of the mutations of surface-exposed residues might influence the interaction with different cellular partners. However, these mutations do not affect the interaction between RPGR and PDEδ (Wätzlich et al., 2013). The patient mutations that seem most likely to influence the RPGR-RID interaction are the RPGR mutations G320R and H324E, given their proximity to the interface (Figure 4A). The K29R and N345D disease-related mutations in RPGR are located in the vicinity of the disordered linker connecting the N-terminal peptide of the RID domain to the start of the C2 domain at residue D1114 (Figure 4B). We thus introduced the four patient mutations into RPGR and isolated the proteins. The G320R mutation led to an insoluble RPGR protein (it introduces a steric clash to the loop containing F271), but the other mutations did not affect the solubility of the protein. Pull-down experiments showed that none of these mutations had any visible effect on binding the RID of RPGRIP1 (Figure 4C).

The RPGRIP1 mutation D1114G found in LCA has been described to abolish the interaction with the RCC1 domain of RPGR (Lu et al., 2005). Based on our crystal structure, this seems highly unlikely, as residue D1114 is not localized near the interface. Pull-downs confirmed that this mutation indeed does not interrupt RPGR binding (Figures 4B and 4D).

**PDEδ and RPGRIP1 Competition**

PDEδ loaded with farnesylated Rheb cargo can dock onto the surface of RPGR to form a stable ternary complex (Wätzlich et al., 2013). An overlay of the RPGR-PDEδ and RPGR-RID complexes, however, shows that PDEδ and RPGRIP1 bound to the
amounts of RIDL222.

(D) A preformed RPGR-RIDL246 complex is incubated with increasing RIDL279 and His-RID222. The last two lanes show binding of RPGR-R323E to (C) A preformed RPGR-RIDL279 complex is incubated with increasing amounts of RID222. The last two lanes show binding of RPGR-R323E to RIDL279 and His-RID222. (D) A preformed RPGR-RIDL246 complex is incubated with increasing amounts of RID222.

surface of RPGR would clash with each other (although the overlap is quite small), indicating that their binding is at least partially exclusive. A ternary complex among RPGR, PDEβi, and RPGRIP1 in which the RID would contact both contact areas of the RPGR β propeller seems sterically not feasible (Figure 4E). However, since the interface areas on the top and side of the propeller are of similar sizes, it is feasible that the PDEβi-cargo complex might weaken, but not interrupt, the RPGR-RPGRIP1 interaction.Biochemical experiments in which we titrated the complex might weaken, but not interrupt, the RPGR-RPGRIP1 interaction. Given that the RPGR mutation R323E abolishes RID222 binding, we investigated its effect on RIDL246 binding. However, pull-downs show that RIDL222 with NFRLPQ no longer interacts with RPGR (Figure 5B). This is consistent with the observed binding mode of the peptide, where V1101 and P1104 latch into two depressions of the RPGR surface (Figure 2E). Mutation to larger residues (N and L in this case) would be expected to disrupt binding. One might argue that replacing the peptide in RIDL246 by the RID sequence could lead to an increased interaction with RPGR. However, pull-downs show that RIDL246-VIVPPM no longer interacts with RPGR (Figure 5B).

To investigate the differences between the RID and RIDL proteins in more detail, we performed competition experiments. We formed RPGR-RIDL complexes by incubating 20 μM RPGR with 60 μM RIDL279 or RIDL246. Then, we added increasing amounts of RID222 (0, 5, 20, 60, and 80 μM for RIDL279; and 0, 5, 20, and 60 μM for RIDL246). Although 60 μM RID222 is sufficient to completely outcompete binding of RIDL246 to RPGR, 80 μM RID222 is required in the case of RIDL279 (Figures 5C and 5D). Hence, the longer N terminus of RIDL279 seems to stabilize the complex. Given that the RPGR mutation R323E abolishes RID222 binding, we investigated its effect on RIDL279 binding as well. As can be seen in Figure 4C, the R323E mutation also diminishes the RIDL279 binding, but the effect is not as pronounced as with RID222. Consequently, it appears that the RID and RIDL proteins bind the RPGR β propeller in a similar fashion, but RID binding is much stronger.

**RPGRIP1 versus RPGRIP1L**

RPGRIP1L is considered a close homolog of RPGRIP1 and has the same domain architecture (Figure 1A). Previous yeast-two-hybrid experiments described binding of the C-terminal domain of RPGRIP1L to RPGR as well (Khanna et al., 2009). To investigate the interaction between RPGR and RPGRIP1L, we created constructs for the C-terminal domain of RPGRIP1L starting from amino acids M1037, L1070, S1109, T1137 and P1145 (Figure 1A), producing RID-like (RIDL) domains of 279, 246, 207, 179, and 171 residues. Pull-downs with Streptagged RPGR show that RIDL279 and RIDL246 bind to the RCC1 domain of RPGR (Figure 5A). A comparison with the RID constructs of RPGRIP1 and their interactions (Figure 1B) demonstrates that the RIDL constructs need to be much longer (the interaction is already very weak with RIDL246) and bind with apparently lower efficiency (Figures 5A, 5C, and 5D).

The sequence alignment of the C-terminal domains of RPGRIP1 and RPGRIP1L shows that residues E1121 and E1245, which are part of the central region of the RPGR-RPGRIP1 interface and form salt bridges to R323, are conserved in RPGRIP1L (Figure S2). However, residue H1174 of RPGRIP1, which seems essential for binding, is not conserved in RPGRIP1L. It is replaced by Asn1202, which in principle could also be involved in a similar type of H-bond donor and/or acceptor interaction with RPGR residues D321 and N336. The most significant difference in interface residues between RPGRIP1 and RPGRIP1L is the N-terminal peptide D1097 SDDVIVPPM of RPGRIP1, which does not seem to have an equivalent in RPGRIP1L. Although sequence homology is very weak in this area, we considered the region (N1117FRLPG) in RPGRIP1L as a potential analog of V1101IVPPM (Figure S2). To get an idea of the importance of this small peptide, we replaced the sequence in RPGRIP1 by the respective RPGRIP1L sequence and vice versa. Pull-downs show that RIDL222 with NFRLPQ no longer interacts with RPGR (Figure 5B). This is consistent with the observed binding mode of the peptide, where V1101 and P1104 latch into two depressions of the RPGR surface (Figure 2E). Mutation to larger residues (N and L in this case) would be expected to disrupt binding. One might argue that replacing the peptide in RIDL246 by the RID sequence could lead to an increased interaction with RPGR. However, pull-downs show that RIDL246-VIVPPM no longer interacts with RPGR (Figure 5B).

**Comparison between the RID of RPGRIP1 and Other C2 Domains**

In general, the sequence similarity between C2 domains is relatively low, which renders correct identification quite difficult. However, by applying several computational methods and in-depth sequence analysis, Zhang and Aravind (2012), were able to recognize several distinct types of C2 domains in ciliary proteins. They identified three different types of C2 domains in both RPGRIP1 and RPGRIP1L. Furthermore, they predicted the middle C2 domain (C2C) to be a classical protein kinase C (PKC)-C2 domain and postulated that the other two C2 domains (C2N and RID) are more divergent. A Dali search using the PDB
file of RID196 as input identified the C2 domain of the human protein Itchy, a E3 ubiquitin protein ligase homolog (PDB ID 2NQ3, rmsd = 2.24 Å over 115 residues) as the closest structural match (Figure S3A). Other close matches include the C2A domain of rat otoferlin (PDB ID 3L9B [Helfmann et al., 2011], rmsd = 2.73 Å over 112 residues; Figure S3B) and the C2N domain of RPGRIP1L (PDB ID 2YRB, rmsd = 2.60 Å over 118 residues; Figure S3C).

The 3D structure of most C2 domains consists of an eight-stranded β sandwich composed of two four-stranded, antiparallel β sheets. The presence of a small α helix between β6 and β7 is highly conserved as well. Superposition of RID196 with the C2 domains of Itchy, otoferlin, and RPGRIP1L shows that they all possess a type II topology and the positions of the β sheets are rather conserved (Figure S3). However, there are big differences in the loop regions. In comparison with the other C2 domains, the β3 strand of RID196 is unusually short (two amino acids compared with four in the human protein Itchy and in otoferlin). Instead, a flexible loop runs in the opposite direction of the β2 strand. Another striking difference between RID222 and the Dali homologs is the presence of two extra α helices—α2 and α4—near α3, where the N and C termini of the molecule come close to each other.

Initially, C2 domains were thought to act as Ca\(^{2+}\)-dependent membrane-binding domains. The Ca\(^{2+}\)-binding pocket is usually composed of three different loops localized at one side of the molecule. Generally, multiple Ca\(^{2+}\) ions are coordinated by interactions with Asp side chains as well as with the protein backbone. Lipid binding can occur via these Ca\(^{2+}\)-binding loops and/or a basic patch in the concave side of the β sandwich, called the β groove. Surface-exposed residues in the C2A-β binding loops determine selectivity for membrane phospholipids such as phosphatidic acid, phosphatidylserine, and phosphatidylinositol, whereas the β groove usually binds different phosphatidylinositol phosphates (PIPs).

Superposition of the crystal structures of RID196 and PKC\(\alpha\) complexed with Ca\(^{2+}\) ions (PDB ID 3GPE) shows that the Asp residues that coordinate the three Ca\(^{2+}\) ions in PKC\(\alpha\) are absent in the RID of RPGRIP1, and only Glu1224 is in a roughly similar position to Asp254 in PKC\(\alpha\). However, it cannot be excluded that the side chains of Glu1222 and Glu1224 and some carbonyl oxygen atoms might nevertheless form a Ca\(^{2+}\)-binding pocket at that position. To determine whether the RID C2 domain binds Ca\(^{2+}\), we performed isothermal titration calorimetry (ITC) experiments, which confirmed that no Ca\(^{2+}\) is bound to the RID of RPGRIP1 under the same conditions in which Ca\(^{2+}\) binding to the C2A domain of synaptotagmin I is observed (Figure S4A).

The crystal structure of PKC\(\alpha\) in complex with PI(4,5)P\(_2\) (PDB ID 3GPE) reveals the interaction of the lipid with the surface-exposed side chains of K197, K209, and K211 (Figure S4B). Superposition of the structures of PKC\(\alpha\) and the RID of RPGRIP1 shows a charge reversal at the positions of K197 and K211, and a helical turn containing L1153 occupies the place of K209. Hence, it seems highly unlikely that PIPs are bound in the β groove of the RID of RPGRIP1. PIP strips in the presence or absence of CaCl\(_2\) did not show any interaction between His\(_6\)-tagged RID222 or RID196 and different phosphoinositides (data not shown). A very weak interaction with phosphatidic acid was observed both with and without CaCl\(_2\). Other C2 domains have been reported to interact with phosphatidic acid in a Ca\(^{2+}\)-independent manner as well. As an example, PKC\(\varepsilon\) presumably binds phosphatidic acid using residues from the loop connecting the β1 and β2 strands and the loop between the β5 and β6 strands (Ochoa et al., 2001).

The β groove of PKC\(\alpha\), the binding site of PI(4,5)P\(_2\), shows a positively charged surface potential (Figure S4C). In contrast, the RID of RPGRIP1 displays a highly negatively charged surface potential at the same position, and the loop connecting β5 and β6 would also sterically hinder PIP binding (Figure S4D). The NMR structure of the C2N domain of RPGRIP1L possesses a neutral to negatively charged β groove (Figure S4E), while a model of the RIDL based on the RID crystal structure suggests that the RIDL possesses an acidic β groove as well. Hence, PIP binding by the C2N and RIDL domains of RPGRIP1L is similarly unlikely.

**DISCUSSION**

**RPGRIP1 and Cargo Trafficking**

We previously presented a model in which PDE\(\varepsilon\) loaded with farnesylated cargo binds to RPGR, and Arl3-GTP binding to that ternary complex releases cargo from PDE\(\varepsilon\) and dissociates the RPGR-PDE\(\varepsilon\) complex (Wätzlich et al., 2013). Here, we show that binding of RPGR to RPGRIP1 and PDE\(\varepsilon\) is at least partially exclusive. RPGRIP1 was previously shown to localize correctly to the connecting cilium of photoreceptors in RPGR\(^{-/-}\) mice, whereas RPGR failed to localize to the connecting cilium in RPGRIP1\(^{-/-}\) knockout mice (Zhao et al., 2003). Hence, RPGRIP1 seems to be the primary docking platform responsible for recruiting RPGR. Although the mechanistic implications are not entirely clear, our competition experiments suggest that the binding of PDE\(\varepsilon\) to RPGR causes weakening of the RPGR-RPGRIP1 complex. Since binding of RPGRIP1 involves two binding sites, only one of which interferes with PDE\(\varepsilon\), cargo unloading of the PDE\(\varepsilon\)-cargo complex might involve a dynamic shift from a two-partite interaction mode to a one-partite mode involving RPGR and RPGRIP1/RPGRIP1L.

**RPGRIP1 versus RPGRIP1L**

The expression of RPGRIP1 seems to be limited to the retina, while RPGRIP1L is more ubiquitously expressed (Arts et al., 2007; Mavlyutov et al., 2002). Mutations in RPGRIP1 always lead to disorders that display a retinal phenotype, whereas mutations in RPGRIP1L lead to ciliopathies with a much broader spectrum of clinical phenotypes. RPGRIP1L has been shown to be a part of multiple ciliary complexes (Arts et al., 2007; Chih et al., 2012; Sang et al., 2011; Williams et al., 2011), which might explain its involvement in a multitude of ciliopathies. The fact that the C2C domains of both RPGRIP1 and RPGRIP1L interact with NPHP4, and both RID and RIDL interact with RPGR suggests that both proteins fulfill a similar function. Our competition experiments indicate that the interaction between RID and RPGR is stronger than the interaction between RIDL and RPGR independently of the RIDL construct used. This may indicate that the RPGR-RPGRIP1 interaction plays a more important role in the retina than in other tissues. Very
speculatively, this might be related to the enormous trafficking rate of proteins (mainly rhodopsin) through the connecting cilium of photoreceptors (Williams, 2002).

Our pull-down experiments with RID constructs of different lengths show that the C2 domain alone is not sufficient to bind RPGR. RID173 and RID185 (starting from amino acids I1102 and D1114, respectively) do not show any interaction with RPGR, which indicates the importance of the extra interaction between the flexible N terminus of RID and the side of the RPGR β propeller. The same seems to be true for RPGRIP1L. Sequence alignments predict the actual C2 domain to start at residue P1145 (Figure S2), but no interaction is observed between RPGR and RIDL207, RIDL179, and RIDL171 (starting from amino acids S1109, T1137, and P1145, respectively). Also, the interaction of RIDL246 with RPGR is weaker than the interaction of RIDL279, which contains a longer N terminus. Since the N-terminal sequence cannot be exchanged between RID and RIDL without losing binding to RPGR, we speculate that the binding interface on the side of the propeller is totally different between RPGRIP1 and RPGRIP1L and cannot be inferred from sequence alignment and modeling.

Ciliary C2 Domains as Protein-Protein Interaction Modules

Transition zone proteins are linked to a broad range of ciliopathies with overlapping phenotypes and seem to form large complexes that together form the ciliary gate. One of the identified subcomplexes is composed of NPHP1, NPHP4, and RPGRIP1L (also called NPHP8) (Sang et al., 2011). Remarkably, all of these proteins are predicted to contain one or more C2 domains. Another complex consists of the three B9 domain proteins (MKS1, MKSR1, and MKSR2) that are involved in Meckel-Gruber syndrome (MKS), a neonatal lethal ciliopathy. B9 domains are ciliary C2 domains that are not expected to bind Ca²⁺ and are present in nearly all ciliated organisms (Bias et al., 2009; Zhang and Aravind, 2010). Furthermore, the MKS-associated protein CC2D2A (= MKS6) is the Joubert-linked protein AhiI are predicted to possess two C2 domains as well (Sang et al., 2011; Zhang and Aravind, 2012). Yeast-two-hybrid experiments have shown that the C2C domains of both RPGRIP1 and RPGRIP1L interact with NPHP4, and in vitro pull-downs further demonstrated that the interaction between the C2C of RPGRIP1 and NPHP4 is Ca²⁺ independent (Roepman et al., 2005). Possibly, some of these C2 domains might serve to anchor the transition zone complexes to the membrane via their interactions with phospholipids. However, based on our findings presented here, we would argue that these C2 domains instead serve as protein-protein interaction modules connecting the different transition zone proteins. Here, we show that the RPGR-interacting domain of RPGRIP1 is a C2 domain as well. Its convex surface constitutes the main interaction site with residues of the connecting loops on the top of the RPGR β propeller.

Although the phospholipid- and Ca²⁺-binding properties of many C2 domains are well characterized, very little is known about how C2 domains mediate protein-protein interactions. One of the few C2 domains that have been shown to act as a genuine protein-protein interaction module is the C2A domain of Munc13-1, which binds to the zinc finger domain of RIM2α (Lu et al., 2006). However, the binding mode is totally different from the one we observe in the RPGR-RPGRIP1 complex.

One of the closest structural homologs of the RID of RPGRIP1 is the C2A domain of otoferlin (Figure S3B). Otoferlin is a multi-C2 domain protein that plays a role in exocytosis in auditory hair cells. Most of its C2 domains have been implicated in Ca²⁺ and phospholipid binding (Johnson and Chapman, 2010). In contrast, the C2A domain does not bind Ca²⁺ or phospholipids and does not possess a positively charged β groove for the interaction with PIPs (Helfmann et al., 2011). The function of the C2A domain has not been elucidated, but it might also serve as a protein-protein interacting domain.

CC2D2A (= MKS6) is a transition zone protein with a domain architecture similar to that found in RPGRIP1 and RPGRIP1L. CC2D2A is predicted to consist of a coiled coil domain followed by two C2 domains and a transglutaminase-like domain (Zhang and Aravind, 2012). Given that CC2D2A has been reported to be present in several transition zone complexes, its C2 domains might serve to interact with other proteins as well. In C. elegans, multiple genes encoding transition zone proteins were disrupted alone and in combination with each other (Williams et al., 2011). Based on the ciliary anomalies observed in these mutant worms, CC2D2A was grouped in a MKS module together with MKS1, MKSR1, MKSR2, and MKS3. RPGRIP1L (NPHP8) was suggested to link the MKS module to an NPHP module composed of NPHP1 and NPHP4. Using tandem affinity purification (TAP) and mass spectrometry, Chih et al. (2012) identified a B9 complex composed of CC2D2A, MKS1, MKSR1, MKSR2, AhiI, TCTN1, TCTN2, KCTD10, TMEM17, and TMEM231. The majority of these proteins contain C2 domains, which makes it likely that specialized C2 domains, such as those identified in this study, serve as the major protein-protein interaction modules in transition zone complexes.

EXPERIMENTAL PROCEDURES

Constructs

Streptagged RPGR (comprising amino acids 1–392) was cloned into the pET28a vector. All RID and RILD constructs were cloned into a pProExHbl vector containing an N-terminal His₆-tag and tobacco etch virus (TEV) cleavage site. Mutagenesis was performed with the QuikChange method (Stratagene).

Protein Purification

Streptagged RPGR was expressed in E. coli BL21(DE3), and His₆-tagged RID and RILD proteins were expressed in E. coli BL21(DE3) codon plus RIL cells. His₆-tagged C2A of synaptotagmin I was expressed in Rosetta(DE3) cells. Bacterial cells were grown in terrific broth medium containing the appropriate antibiotics at 25°C. Cells were induced with 100 μM isopropyl β-D-1-thiogalactopyranoside and grown overnight at 18°C to allow protein production. Streptagged RPGR was purified over a Streptactin column, followed by size-exclusion chromatography. His₆-tagged RID and RILD proteins were purified over a His-Trap or Talon column, followed by size-exclusion chromatography. When necessary, the His₆-tag was removed by TEV cleavage overnight at 4°C. The final buffer for all proteins contained 50 mM Tris pH 8.0, 50 mM NaCl, 5% glycerol, and 1 mM TCEP. The His₆-tagged C2A domain of synaptotagmin I was purified in exactly the same way as the RID and RILD proteins.

Crystallization, X-Ray Structure Solution, and Refinement

To set up crystal screens, 450 μM Streptagged RPGR and 675 μM untagged RID196 were mixed in a 1:1 ratio. The best-diffracting crystals were obtained.
through the hanging-drop vapor diffusion method in a condition containing 300 mM MgCl₂, and 18% PEG-3350. Crystals containing the complex of RPGR and semelomethionine-substituted RID196 were obtained in the same condition. Crystals were flash-frozen using the mother liquor containing 25% of glycerol as cryoprotectant. Semelomethionine single-wavelength anomalous diffraction (SeMet SAD) and native X-ray data were recorded at the Swiss Light Source (Villigen, Switzerland) at beamline PXII. Data were processed using XDS (Kabsch, 2010) in space group P2₁2₁2₁ at a resolution of 1.83 Å for the SeMet SAD data. Because the quality of the native crystals was worse, the SeMet data were used for refinement and model building. The crystal structure was solved combining phases obtained by molecular replacement using the RCC1 domain of RPGR (PDB ID 4JHN) as template and by SAD from semelomethionine-incorporated RID196. These steps were carried out using AutoSol (Terwilliger et al., 2009) as part of Phenix (Adams et al., 2010). This final model was built with Coot (Emsley and Cowtan, 2004) and refined with Phenix and pII-tagged RPGR were added to Streptactin beads. After a 1 hr incubation at 4°C, the mixtures were incubated for 2 hr at 4°C and washing of the beads, increasing amounts of PDE/C₁₄ and the elutions were loaded onto an SDS-PAGE gel for analysis.

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K.R. performed biochemical and crystallographic experiments. M.B. and I.R.V. assisted with the crystallography. K.R. and A.W. designed experiments, analyzed data, and wrote the manuscript.
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