Structural basis for Arl3-specific release of myristoylated ciliary cargo from UNC119

Shehab A Ismail¹, Yong-Xiang Chen²,
Mandy Miertzschke¹, Ingrid R Vetter¹,
Carolin Koerner¹ and Alfred Wittinghofer^{1,*}

¹Structural Biology Group, Max Planck Institut für Molekulare Physiologie, Dortmund, Germany and ²Department of Chemistry, Key Lab of Bioorganic Phosphorus Chemistry and Chemical Biology (Ministry of Education), Tsinghua University, Beijing, China

Access to the ciliary membrane for trans-membrane or membrane-associated proteins is a regulated process. Previously, we have shown that the closely homologous small G proteins Arl2 and Arl3 allosterically regulate prenylated cargo release from PDE δ . UNC119/HRG4 is responsible for ciliary delivery of myristoylated cargo. Here, we show that although Arl3 and Arl2 bind UNC119 with similar affinities, only Arl3 allosterically displaces cargo by accelerating its release by three orders of magnitude. Crystal structures of Arl3 and Arl2 in complex with UNC119a reveal the molecular basis of specificity. Contrary to previous structures of GTP-bound Arf subfamily proteins, the N-terminal amphipathic helix of Arl3•GppNHp is not displaced by the interswitch toggle but remains bound on the surface of the protein. Opposite to the mechanism of cargo release on PDE δ , this induces a widening of the myristoyl binding pocket. This leads us to propose that ciliary targeting of myristoylated proteins is not only dependent on nucleotide status but also on the cellular localization of Arl3.

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Introduction

Primary cilia are microtubule-based antenna-like organelles that are found on almost all human cells. They are hubs of many developmental signalling pathways, including sonic hedgehog and Wnt signalling (Eggenchwil and Anderson, 2007; Corbit *et al*, 2008; Goetz and Anderson, 2010). Defects in ciliary function result in a copious spectrum of diseases collectively called ciliopathies (Badano *et al*, 2006; Novarino *et al*, 2011). Since the protein synthesis machinery is located outside the ciliary compartment, and access to the compartment is restricted, protein targeting into and trafficking inside cilia are instrumental for proper formation and

functioning of cilia (Pazour and Rosenbaum, 2002; Rosenbaum and Witman, 2002).

PDE δ (delta subunit of phosphodiesterase) also called PrBP (prenyl binding protein), UNC119a (uncoordinated) also called HRG4 (human retina gene 4) and UNC119b are homologous proteins that share a similar structural fold, an immunoglobulin like β -sandwich, with RhoGDI (Higashide *et al*, 1996; Hanzal-Bayer *et al*, 2002; Zhang *et al*, 2007). For PDE δ , UNC119a and UNC119b, it has been shown that they contain a hydrophobic pocket that accommodates lipid moieties of post-translationally modified membrane-associated proteins (Hanzal-Bayer *et al*, 2002; Ismail *et al*, 2011; Wright *et al*, 2011; Zhang *et al*, 2011). They thus act as solubilizing factors for lipidated cargo which is why they have been labelled ‘GDI-like solubilizing factors’ (Ismail *et al*, 2011; Chandra *et al*, 2012). These GDI-like solubilizing factors have been implicated in ciliary trafficking of lipidated proteins.

PDE δ has been shown to shuttle prenylated cargo-like Ras and Rheb between membrane compartments and is believed to be important for transporting proteins such as Rhodopsin kinase (GRK1) and rod phosphodiesterase subunits to outer segments of photoreceptors, a specialized sensory primary cilium (Zhang *et al*, 2007; Chandra *et al*, 2012). UNC119a and UNC119b, which share 55% sequence identity, have been reported to be critical for targeting and trafficking myristoylated proteins into cilia. UNC119a recognizes and is instrumental for trafficking of Transducin- α in photoreceptors (GNAT-1) and the G α subunits ODR-3 and GPA-13 in *C. elegans* olfactory cilia (Zhang *et al*, 2011). In another recent study, it was demonstrated that UNC119a and UNC119b recognize several myristoylated proteins including the ciliary protein cystins and NPHP3, the latter one being involved in the ciliopathy nephronophthisis (Wright *et al*, 2011).

The GDI-like solubilizing factors PDE δ and UNC119a/b also bind to the small G proteins Arl3 and Arl2 in a nucleotide-dependent manner, with similar affinities, and hence can be considered as their effectors (Linari *et al*, 1999; Hanzal-Bayer *et al*, 2002; Kobayashi *et al*, 2003; Veltel *et al*, 2008b). Despite sharing several effectors, Arl3 and Arl2 are biologically distinct and seem to regulate different processes (Zhou *et al*, 2006; Gillingham and Munro, 2007). Arl3 is exclusively found in ciliated organisms, present in cilia and is specifically regulated by the GTPase Activating Protein (GAP) RP2, a protein mutated in X-linked retinitis pigmentosa (Schwahn *et al*, 1998; Avidor-Reiss *et al*, 2004; Veltel *et al*, 2008a). Furthermore, Arl3 knockout mice showed manifestations typical for ciliopathies such as photoreceptors degeneration and renal cysts (Schrack *et al*, 2006). We have recently shown that Arl2 and Arl3 act as GDI-like displacement factors (GDFs) for PDE δ by allosterically displacing its prenylated cargo (Ismail *et al*, 2011). By binding to PDE δ they induce a closed conformation which is no longer able to bind cargo. In a recent study, it was reported that myristoylated cystin is released from UNC119 by Arl3 and not by Arl2 (Wright *et al*, 2011). However, the molecular mechanism of the release

*Corresponding author. Department of Structural Biology, MPI für Molekulare Physiologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany. Tel.: +49 231 133 2100; Fax: +49 231 133 2199; E-mail: alfred.wittinghofer@mpi-dortmund.mpg.de

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as well as the structural requirements for the specificity remains elusive.

Here, we show that Arl3 but not Arl2 allosterically displaces myristoylated cargo by the formation of a fast dissociating ternary complex and accelerates the release by three orders of magnitude. By solving the structures of Arl3•GppNHp and Arl2•GppNHp in complex with UNC119a we show that the unique conformational change of the interswitch toggle of Arl3 and the position of the N-terminal amphipathic α -helix are the hallmarks of the specific release mechanism. Surprisingly, the release mechanism on UNC119a is entirely different from that of PDE δ in that Arl3 binding leads to a more open rather than a more closed conformation.

Results

UNC119 cargo is specifically released by Arl3•GppNHp

Using fluorescence polarization measurements, we first determined the dissociation constants of UNC119a binding to fluorescein-labelled myristoylated N-terminal peptides of its known interactors. For Transducin- α (GNAT-1) and NPHP3, affinities were determined to be 7 and 14 nM, respectively (Supplementary Figure 1). Affinities may in fact be higher since the binding curve is somewhat linear and resembling more an active site titration. Furthermore, we determined the affinity to the *C. elegans* G α subunit ODR-3 to be 600 nM. Although the specificity for GNAT1 compared to ODR-3 is similar to what has been reported before the affinities are higher, most likely due to the different method used and the fact that for ODR-3 the lauroylated (C12) instead of the myristoylated (C14) peptide, was used (Zhang *et al*, 2011). The dependence of the binding affinity on the sequence of the myristoylated N-terminus is different from that of PDE δ that binds farnesylated peptide almost independently of the sequence, and where the structure has shown that binding of the C-terminal residues is exclusively via main chain interactions (Ismail *et al*, 2011; Zhang *et al*, 2011). Although UNC119a and UNC119b share 55% sequence identity it was reported that knocking down of UNC119b but not UNC119a impairs the ciliary localization of NPHP3 (Wright *et al*, 2011). To investigate this observation, we also determined the affinity of UNC119b to NPHP3 and GNAT-1. Binding to UNC119b was very similar with Kds of 8 and 27 nM, respectively (Supplementary Figure 1).

To test the effect of Arl3 and Arl2 on cargo-loaded UNC119, Arl3•GppNHp or Arl2•GppNHp was added to a preformed complex of UNC119a and fluorescently labelled myristoylated NPHP3 and GNAT-1 peptides. As shown earlier for cystin by a solid phase binding assay, Arl3•GppNHp but not Arl2•GppNHp was able to partially disrupt both the UNC119a–GNAT1 and UNC119a–NPHP3 complexes as seen by a decrease in the fluorescence polarization signal (Figure 1A and C; Wright *et al*, 2011). This shows that under the standard conditions used there is equilibrium between cargo-bound and cargo-free UNC119 complexes. Arl3•GDP did not induce this effect supporting the notion that UNC119 is an Arl3 effector that requires the GTP-bound conformation for binding (Supplementary Figure 1d). The inability of Arl2•GppNHp to displace myristoylated cargo was not due to a weaker binding, as we have reported before that the affinity of UNC119a to Arl2 is in fact very similar to that of Arl3 (Veltel *et al*, 2008b).

Similar effects were seen for the interaction of UNC119b complexes, which also showed specificity for Arl3 (Figure 1B and D). However, UNC119b seems to be more prone to cargo release than UNC119a. Under the conditions used, the release by Arl3•GppNHp of the GNAT1 peptide was almost 100% for UNC119b, but only 60% for UNC119a and a similar large difference is observed for the NPHP3 peptide. Furthermore, a limited release of myristoylated peptide by Arl2•GppNHp could be noticed only in case of UNC119b. This supports the observation of the different effects of knockdown of UNC119a versus UNC119b on ciliary localization of NPHP3, which is much more significant for UNC119b (Wright *et al*, 2011).

Allosteric mechanism of cargo release from UNC119a/b

We then wanted to test whether the disruption of the cargo complex is due to a pure competition mechanism where the dissociation of UNC119-loaded cargo is a prerequisite for Arl3•GppNHp binding, or whether Arl3•GppNHp is able to bind to the UNC119-cargo complex and actively releases its cargo via the formation of a low affinity ternary complex. The former mechanism would mean that the dissociation rate constant is similar in presence and absence of the small G protein whereas the later mechanism would be reflected by an increase of the dissociation rate constant of myristoylated cargo. To measure the rate constant of peptide release from UNC119a, we used a polarization-based stopped flow experiment. A 100-fold excess of non-labelled GNAT-1 peptide was added to a preformed complex between 1 μ M fluorescein-labelled N-terminal myristoylated GNAT-1 peptide and 1 μ M UNC119a. The dissociation rate constant for the fluorescent peptide is 0.003 s^{−1} which agrees with the high binding affinity we observe by equilibrium measurements. Strikingly in the presence of Arl3•GppNHp the rate constant increased 1233-fold (3.7 s^{−1}) (Figure 1E). On the other hand, addition of Arl2•GppNHp resulted in only a modest 14-fold increase (0.048 s^{−1}) arguing that Arl2 also forms a ternary complex but is lacking an efficient displacement machinery (Figure 1F).

From the above, we conclude that although Arl2•GppNHp and Arl3•GppNHp can bind to UNC119 in complex with myristoylated cargo only Arl3 is able to effectively displace cargo by an allosteric mechanism. In order to further investigate the mechanism of release and why this mechanism, in the face of the high similarity between Arl2 and Arl3 and their similar binding affinity, is exclusive to Arl3 we set out to solve the crystal structures of Arl3 and Arl2 in complex with UNC119a.

Crystallization and overall structure

Crystallization of Arl2 or Arl3 in complex with UNC119a using full-length proteins was not successful. We then performed a limited tryptic digestion of Arl3 and Arl2 bound to GppNHp in the presence and absence of UNC119a. UNC119a was digested into a stable smaller fragment that can still bind the G proteins (data not shown). Although we could not observe a difference in electrophoretic migration for Arl3 and Arl2 after digestion, N-terminal sequencing nevertheless showed that in absence of UNC119a, the N-terminal residues of both Arl2 and Arl3 were removed by proteolysis. In the case of Arl3•GppNHp, however, the N-terminus is partially protected from digestion by the presence of UNC119 as

Figure 1 Cargo release from UNC119 by Arl3•GppNHp. (A–D) Fluorescence polarization measurement where $0.5\ \mu\text{M}$ UNC119a (A/C) or UNC119b (B/D) is added to a solution of $0.7\ \mu\text{M}$ fluorescein-labelled myristoylated N-terminal GNAT1 peptide (A/B) or $0.5\ \mu\text{M}$ fluorescein-labelled myristoylated N-terminal NPHP3 (C/D) followed by the addition of $3.8\ \mu\text{M}$ full-length Arl3•GppNHp or $3.8\ \mu\text{M}$ full-length Arl2•GppNHp. Addition time points are indicated by arrows. (E, F) Stopped flow fluorescence polarization kinetic experiment where a complex between $1\ \mu\text{M}$ fluorescein labelled N-terminal myristoylated GNAT-1 and $1\ \mu\text{M}$ UNC119a was mixed with 100-fold excess unlabelled peptide and $10\ \mu\text{M}$ Arl2/3 as indicated.

demonstrated by the presence of a mixture of digested and full-length protein (not shown). Since this was not observed for Arl2, we conclude that the N-terminal helix of Arl3, but not Arl2 plays a role in the interaction with UNC119a.

Since the N-terminal helix was highly flexible in the previous structures of Arl2 with either PDE δ or BART and crystallization required low amounts of trypsin, we set up crystallization trials with truncated Arl2, residues 17–178 (ΔArl2 from now), in complex with UNC119a (Hanzal-Bayer *et al*, 2002; Zhang *et al*, 2009). Since our biochemical assays showed the N-terminal helix to be important for the release by Arl3 (see below) and it was at least partially protected from proteolysis we used full-length Arl3 in complex of UNC119a. We obtain well diffracting crystals for the complexes with $\Delta\text{Arl2}\bullet\text{GppNHp}$ and Arl3•GppNHp, with resolutions of 2.6 and 2.1 Å, respectively. The structures were solved by molecular replacement using Arl3•GppNHp from

the $\Delta\text{Arl3}\bullet\text{GppNHp}\bullet\text{RP2}$ complex structure (PDB 3BH6), Arl2 from Arl2•GTP•PDE δ (PDB 1KSH) and UNC119a (PDB 3GQQ) as search models. In case of the Arl3•GppNHp complex, the asymmetric unit contained four molecules. For the Arl2•GppNHp complex, a pseudotranslation symmetry was detected and the asymmetric unit contained two complexes. The structures showed a typical G protein fold for Arl2 and Arl3 and a β -sandwich immunoglobulin fold for UNC119a (Figure 2A and B).

The first 59 amino acids of UNC119a could not be found in the electron density most likely due to proteolytic digestion to a smaller fragment. Electron density of Arl3•GppNHp including an extra electron density for the N-terminal helix was clearly seen supporting the observation from proteolytic digestion that the N-terminal helix is stabilized in the Arl3 complex. The interaction with UNC119a by both Arl2 and Arl3 was mediated, as in case of PDE δ , primarily by $\beta 2$ from

Figure 2 Overall structure and interface of Arl–UNC119a complexes. (A, B) Ribbon representation of overall structure of full-length Arl3•GppNHp in blue (A) or truncated Arl2•GppNHp, in yellow (B) in complex with UNC119a in grey, with GppNHp as ball-and-stick. The switch regions are shown in salmon, the interswitch toggle in cyan and the Arl3 N-terminal helix in red. (C, D) Schematic diagram of the interacting residues, colour code of residues is as in (A, B). Polar interactions are represented as red dotted lines and non-polar interactions as black dotted lines. Interacting residues only observed in Arl3•UNC119a interface are marked with an asterisk and residues involved in main chain interactions are underlined.

Arl and $\beta 7$ from UNC119a forming a continuous β -sheet with typical main-chain interactions (Hanzal-Bayer *et al*, 2002). The buried surface areas of the complexes were 707 Å² and 931 Å² for Arl2 and Arl3, respectively. The interaction involved both hydrophobic and polar interactions; as shown schematically in Figure 2C and D. Residues from switch I, II and the interswitch region constitute the interaction interface, which would explain the nucleotide specificity for binding. Arl3•GppNHp showed more extended interaction interface mainly due to less ordered switch I in case of Arl2 and more interactions in the interswitch region (see below).

Proteins of the Arf subfamily of small G proteins possess an N-terminal amphipathic helix and many have been shown to be myristoylated (Gillingham and Munro, 2007). The amphipathic helix together with the myristoyl group is believed to stabilize membrane localization/interaction, particularly in the GTP-bound conformation. Nevertheless in some cases the amphipathic helix has been shown to be sufficient for membrane binding as in case of Sar1 and Arl6 (Lee *et al*, 2005; Jin *et al*, 2010). Although Arl2 and Arl3 have, in

addition to an amphipathic helix, a conserved glycine at position 2, both proteins are not predicted to be myristoylated and experiments to show such post-translational modification have failed (Bologna *et al*, 2004; Gillingham and Munro, 2007).

When Arf/Arl proteins are bound to GDP the amphipathic helix is located in a hydrophobic pocket on the surface of the protein and the β -strands $\beta 2$ and $\beta 3$ are shifted by two residues relative to their position in other small G proteins (Goldberg, 1998; Hillig *et al*, 2000; Menetrey *et al*, 2000). A common and unique structural feature for Arf subfamily proteins, also found for Arl2/3, is that these β -strands present between switch I and II, termed the interswitch toggle, undergo a two amino-acid register shift upon binding to GTP such that they now occupy the canonical position found in all other G proteins (Goldberg, 1998; Pasqualato *et al*, 2001; Vetter and Wittinghofer, 2001; Hanzal-Bayer *et al*, 2002). This register shift results in the movement of the interswitch toggle and displaces the amphipathic helix out of the hydrophobic pocket, making it available for binding to membranes (Goldberg, 1998;

Figure 3 A new Arl3 toggle conformation. The interswitch toggle and the N-terminal helix shown after superimposition of the G domains in different complexes (A) Arl3•GDP (yellow) versus ΔArl2•GppNHp (grey) from the Arl2•UNC119a complex. (B) Arl3•GppNHp (cyan) from the UNC119a complex versus Arl2 from the PDEδ complex. (C) Arl3•GppNHp (cyan) versus ΔArl2•GppNHp (grey) from their UNC119a complexes. (D) Arl3•GppNHp (cyan) from the UNC119a complex versus Arl3•GDP (yellow). Red arrows indicate the conformational changes.

Pasqualato *et al*, 2001). This feature is unique for Arf/Arl family proteins as it connects the nucleotide status to the membrane binding capacity of Arf proteins. The structure of Arl2•GppNHp in the UNC119a complex shows the canonical GTP-dependent conformation where the β-turn of the interswitch toggle fills the hydrophobic pocket on the surface that was previously occupied by the amphipathic helix in Arl•GDP (taking Arl3•GDP as a homologous structure; Figure 3A). Except for the presence of the N-terminal helix, this structure is very similar to that of full-length Arl2 in complex with PDEδ and in conjunction with the structure of Arl3•GDP recapitulates the Arf subfamily conformational change (Figure 3A and B; Hillig *et al*, 2000; Hanzal-Bayer *et al*, 2002). Based on the conformation

of the N-terminal helix of Arl2 bound to PDEδ and our proteolytic digestion data, we can assume that the N-terminal helix of Arl2 in the UNC119 complex is also pointing into solution.

In case of the complex with full-length Arl3•GppNHp, the typical two-residue register shift also takes place. However, strikingly, the β-turn now moves away from the hydrophobic pocket by making an ~30 degree movement in the plane perpendicular to the β-sheet and towards UNC119a (Figure 3B and C). This movement leaves the hydrophobic pocket on the surface of the protein free to be occupied by the amphipathic helix. The helix thus stays in a position similar as that of the GDP-bound conformation and is not exposed to solution (Figure 3D) as shown for Arl2-PDEδ and Arl2-BART struc-

tures and assumed for the other Arf/Arl subfamily structures (Goldberg, 1998; Pasqualato *et al*, 2001; Hanzal-Bayer *et al*, 2002; Zhang *et al*, 2009). This structural observation is in agreement with the proteolytic data.

Release mechanism

When the structure of the Arl3–UNC119a complex is superimposed on that of UNC119a in complex with N-lauroylated transducin peptide (Figure 4A) we can see that UNC119a is in a very similar conformation in both complexes (Zhang *et al*, 2011). We also note that the entrance to the hydrophobic pocket is not overlapping with the binding site of Arl2/3 (Figure 4A). Thus, it seems that a ternary complex of Arl2 and Arl3 with cargo-bound UNC119a is structurally feasible and thus agrees with an allosteric regulation of cargo release. We have shown previously that Arl2 and Arl3 regulate cargo binding to PDE δ by closing the hydrophobic pocket such that farnesylated peptides and proteins can no longer bind (Ismail *et al*, 2011). Thus, we wanted to know if UNC119 is regulated in a similar manner. Since loop λ 9 of UNC119, which is part of the hydrophobic pocket, is disordered in the Arl2 complex, and since Arl3 but not Arl2 is releasing cargo, Arl3•GppNHp•UNC119a is used to examine the hydrophobic pocket. In this structure loop λ 9 is well ordered, but compared to the cargo complex there is a slight movement

away from the hydrophobic pocket (Figure 4B). As a result of this and further changes the hydrophobic pocket of UNC119a in the Arl3 complex is more open compared to that of the UNC119a–lauroylated GNAT-1 complex. In total, the lipid binding cavity expands from 575 Å³ to 657 Å³ (Figure 4C and D) which appears to be sufficient for significantly weakening of the binding affinity.

A residue that contributes to the formation of the hydrophobic pocket and interacts with cargo is the strictly conserved Tyr194^(UNC119a) (Figure 4B). Tyr194^(UNC119a) undergoes a large main chain shift of 4 Å and a much larger side chain movement away from the hydrophobic pocket upon binding to Arl3GppNHp, resulting in an opening of the pocket (Figure 4B–D). To verify the mechanism and the contribution of Tyr194^(UNC119a), we mutated the latter to Ala. Although the mutant bound to Arl3GppNHp with very similar affinity as compared to wild type (300 nM versus 270 nM, respectively; Supplementary Figure 2a), this mutation impairs the binding of cargo as shown by a 140-fold decrease in affinity for myristoylated peptide (Supplementary Figure 2b). Furthermore, Phe207^(Un119b), which corresponds to Phe196^(UNC119a), was reported to be critical for binding lipidated cargo (Wright *et al*, 2011). Here, Phe196^(UNC119a) located in λ 9 undergoes a 2-Å main chain movement that would lead to a less tight interaction of the side chain with the lipid moiety (Figure 4B).

Figure 4 Cargo binding conformational change. (A) Superimposition (using UNC119) of Arl3•GppNHp•UNC119a (Arl3 in blue/cyan and UNC119a in grey) on UNC119a in complex with lauroylated GNAT-1 N-terminal peptide (UNC119a in yellow and the lauroylated transducin peptide in red) showing the possibility of making a ternary complex. (B) A blow up view of (A), after a 180° horizontal rotation, highlighting the movement of λ 9, Tyr194, Phe196 and Phe137 (stick representation). (C, D) Surface presentation of the lipid binding pockets showing a larger pocket in the complex with Arl3•GppNHp (D) than in complex with peptide (C), with Tyr194 shown as sticks.

Furthermore, this residue is also involved in a π - π T-shaped interaction with Phe137^(UNC119a) which in turn is interacting with the lipid moiety. The movement of Phe196^(UNC119a) as seen in the structure of Arl3•GppNHp•UNC119a would weaken the T-shaped interaction and hence destabilize the interaction of Phe137^(UNC119a) with the lipid. All these rearrangements upon binding Arl3 to UNC119a would loosen the hydrophobic interactions with lipidated cargo. In summary, in spite of the close homology between PDE δ and UNC119 and a similar binding mode for Arl2 and Arl3, the molecular mechanism of cargo release from UNC119a (see Supplementary movie) is opposite to that of PDE δ (Ismail *et al*, 2011).

The Arl3 N-terminal helix as a pocket opener

The most intriguing question was why Arl3 specifically releases UNC119a bound myristoylated cargo, but Arl2 does not. By superimposing the two complex structures the most drastic conformational difference is the interswitch toggle movement (Figure 3C). This movement, which involves β 2 and β 3 of Arl3, results in a more extended β sheet interaction between β 2 from Arl3 and β 7 from UNC119a. Contacts are made by main chain interactions between Gln57^(Arl3) and Leu193^(UNC119a) and Asp195^(UNC119a) in addition to the interaction between Asp195^(UNC119a) with the carbonyl of Ser58^(Arl3) (Figure 5A and B). This would result in the stabilization of Tyr194^(UNC119a) away from the hydrophobic binding pocket and also to the ordering and the movement of residues 195–198 from λ 9 (Figure 4B). These interactions cannot be seen in case of the Arl2–UNC119a complex in which the flexibility of λ 9 is reflected by poor electron density (Figure 5B). The presence of λ 9 in Arl2–UNC119a was confirmed by mass spectrometry after tryptic digestion. However, we would expect that in the Arl2–UNC119a-cargo complex, the ligand binding pocket remains in the closed conformation.

Since the location of the N-terminal helix in the Arl3 complex and the protease digestion studies suggest the importance of the N-terminal helix, we thus wanted to investigate its role in the allosteric regulation. Since Arl2 in the structure lacks the N-terminal helix we compared the structure of Arl3•UNC119a to that of Arl2•PDE δ determined with full-length Arl2. In the Arl2•PDE δ structure, the N-terminal helix is in an exposed conformation pointing into solution (Figure 3B). To test for the importance of the

β -turn conformation and the position of the α -helix and their role for the release mechanism, we measured cargo release by truncated (minus the first 17 residues, Δ Arl3 from now) Arl3•GppNHp on the UNC119a–myristoylated peptide complex. Binding studies show a similar affinity for the truncated versus full-length protein (Supplementary Figure 2c). However, Δ Arl3•GppNHp did not release myristoylated cargo from UNC119a. The moderate increase in the polarization signal after addition of Δ Arl3 indicates the formation of a ternary complex (Figure 6A). Furthermore, the kinetic studies show that Δ Arl3•GppNHp behaved similarly to Arl2 in that both induce a slower increase in the dissociation of cargo (0.025 s^{-1}) (Figure 6C, compare with Figure 1F).

We have shown above that the Y194A^(UNC119a) mutant has a considerable reduced affinity to myristoylated peptide. When we tested cargo release we found, as expected, that Arl3 efficiently releases myristoylated GNAT1 peptide cargo from this weak affinity mutant (Figure 6B). We tested the ability of the truncated Arl3 to release cargo from Y194A^(UNC119a). Although not as efficient as the full-length protein the truncated form showed a decrease in polarization signal. With the mutant, even Arl2•GppNHp is able to at least partially induce release of cargo arguing that only tight binding cargo requires Arl3 and its N-terminal helix for release.

From the structure of the Arl2 complex it appeared that the more conventional location of the interswitch β turn is further stabilized in its position by the interaction to the C-terminal helix, as seen by the His57^(Arl2) and Arg58^(Arl2) interactions with Asp170^(Arl2) and Asp174^(Arl2), respectively (Supplementary Figure 3a). We reckoned that a more flexible β turn would likely aid in UNC119a-cargo release. We thus designed a reverse charge mutation (R58E) for Arl2. Indeed the mutant, unlike the wild type was able to show a slight release of UNC119 cargo (Supplementary Figure 3). Furthermore, the rate of release in the presence of Arl2(R58E)•GppNHp is increased 66-fold as compared to 14-fold for wt Arl2 (Supplementary Figure 3c). This indicates that the stable position of the β -turn does indeed contribute to the inability of Arl2 to mediate release.

The first 22 amino acids of Arl3 (21 in Arl2) which include the N-terminal amphipathic helix and the loop (L1) connecting it to β 1 are divergent between Arl2 and Arl3. In order to test if the protein sequence in this region plays a crucial role in the allosteric mechanism and hence critical

Figure 5 Structural bases of specificity. (A, B) Ribbon representation of the β sheet formed between β 2 and β 7 of Arl3•GppNHp (A) and Δ Arl2•GppNHp (B) in the complexes with UNC119a, with colours as in Figure 2A and B, Ser55, Gln57 and Ser58 from Arl3, Thr54 and Glu56 from Arl2 and Tyr194 and Asp195 of UNC119a are shown as sticks.

Figure 6 The Arl3 N-terminal helix is critical for release. (A) Fluorescence polarization measurement using 0.7 μ M fluorescein-labelled myristoylated N-terminal GNAT1 peptide. At the indicated time points (arrows), 0.5 μ M UNC119a and 3.8 μ M full-length and truncated Arl3•GppNHp are added successively. (B) As in (A) adding 1.5 μ M mutant UNC119a(Y194A) followed by the addition of 3.8 μ M Arl3•GppNHp, or 3.8 μ M Arl2•GppNHp or 3.8 μ M Δ NAr13•GppNHp as indicated. (C) Stopped flow fluorescence polarization experiment where a preformed complex between 1 μ M fluorescein-labelled N-terminal myristoylated GNAT1 and 1 μ M UNC119a was mixed with excess unlabelled peptide and 10 μ M Arl3 or Δ Arl3 as indicated. (D) Fluorescence polarization with the fluorescent peptide as in (A, B) where 0.5 μ M UNC119a or 1.5 μ M UNC119(Y194A) is added to a solution of 0.7 μ M fluorescein-labelled myristoylated N-terminal GNAT1 followed by the addition of 3.8 μ M of the chimeric N2-Arl3•GppNHp, as indicated.

for specificity, we designed a chimeric protein where we exchanged the Arl3 N-terminal 22 amino acids, with the 21 amino acids of Arl2 (N2-Arl3 from now). The affinity of this chimeric protein to UNC119a was very similar to that of either wild-type Arl3 or Arl2 (480 nM) (Supplementary Figure 3c). We then tested the ability of this chimera to release the UNC119a cargo. Unlike wild-type Arl3•GppNHp, N2-Arl3•GppNHp was completely unable to disrupt the UNC119a–GNAT1 complex (Figure 6D). To verify the integrity of the chimeric protein, we tested it against the release sensitive mutant Y194A^(UNC119a). The chimeric protein was indeed able to mediate release, and the effect was similar to that of Arl2 (Figure 6D, compare to Figure 6B). We then constructed an opposite chimera where we exchanged the Arl2 N-terminal 21 amino acids with the 22 amino acids of Arl3 (N3-Arl2). This chimera was not able to release the UNC119a cargo. This indicated that the identity of the Arl3 N-terminal helix is indispensable but not sufficient for cargo release and that other factors contribute to the conformation of the N-terminal helix (e.g., the flexibility of the interswitch β -turn, see above).

Discussion

Arf proteins are characterized by having an N-terminal amphipathic helix which in many members is myristoylated. Although both motifs seem to be required for stable membrane interaction the amphipathic helix can be sufficient for

membrane localization, as shown for Sar1 and Arl6 (Lee *et al*, 2005; Resh, 2006; Gillingham and Munro, 2007; Jin *et al*, 2010). Both Arl2 and Arl3 have in addition to the N-terminal amphipathic helix a totally conserved glycine in position 2 however they are apparently not myristoylated (Bologna *et al*, 2004). Although not much is known about membrane interactions of Arl2 and Arl3 a recent study has shown that the constitutively active Arl3 fractionates to both soluble and membranes fractions which is most likely due to the weak affinity to membranes (Wright *et al*, 2011).

Our study shows that in the GTP-bound state, the amphipathic helix of Arl3 is flexible and remains at least partially attached to the hydrophobic groove in presence of UNC119. Furthermore, it is critical for the allosteric regulation of cargo release from UNC119 that is different for the N-terminal helix of Arl2. This implies that membrane-bound Arl3GTP should not be able to release the cargo. Although the membrane of cilia is a continuous projection from the plasma membrane the lipid and membrane composition is different (Tyler *et al*, 2009; Emmer *et al*, 2010; Rohatgi and Snell, 2010). Ciliary membranes are rich in cholesterol and sphingolipids and hence predicted to be more ordered than the bulk plasma membrane (Tyler *et al*, 2009; Emmer *et al*, 2010). For Arl1 it has been shown that the N-terminal amphipathic helix does not favour lipid ordered domains (Manneville *et al*, 2008). Due to the ciliary membrane composition and the different structure and dynamics of the Arl3 N-terminal helix, it is tempting to speculate that Arl3•GTP will bind weaker, if at

Figure 7 Model for Arl3•GTP localization-dependent UNC119-cargo release. Arl3 affinity to membranes is modulated by its nucleotide status. When bound to GTP the N-terminal amphipathic helix is exposed to solution and is available for membrane binding. The UNC119-cargo release is dependent on the availability of Arl3 N-terminal amphipathic helix. Ciliary membrane is ordered and we speculate that Arl3 amphipathic helix affinity to ciliary membranes would be weakened, hence Arl3 N-terminal amphipathic helix will be available for cargo unloading in cilia. Finally Arl3GAP, RP2, can recruit Arl3•GTP•UNC119 catalysing the GTP hydrolysis to release UNC119 and Arl3GDP.

all, to ciliary membranes. Thus, the Arl3-mediated release of UNC119 cargo may not only dependent on the nucleotide status but also the cellular localization of the protein, thus adding another layer of regulation. We propose a targeting model whereby Arl3•GTP is able to release UNC119-bound cargo only inside cilia where the amphipathic helix is not able to bind to highly ordered membranes and hence available for the allosteric regulation (Figure 7).

An additional complexity arises due to RP2, which is an Arl3-specific GAP known to be myristoylated and localized to plasma and ciliary membranes (Veltel *et al*, 2008a; Hurd *et al*, 2011). Our model suggests a non-membrane bound localization of the Arl3–UNC119 complex since the amphipathic helix is not available for membrane localization. We have shown previously that unlike most small G proteins and their cognate GAPs and effectors, Arl3•GTP, its GAP RP2 and its effector UNC119 are able to form a ternary complex (Veltel *et al*, 2008b). This means that RP2 will be able to recruit the UNC119–Arl3.GTP complex, hydrolyse GTP and thus release UNC119 (Figure 7). Such a mechanism would compensate for Arl3 not co-localizing with its cognate GAP in the membrane.

Materials and methods

Plasmids and proteins

C-terminal Histidine tagged full-length Arl2 and Arl3 constructs as well as GST-tagged truncated Arl2 and Arl3, UNC119a were prepared and proteins were purified as described before (Veltel *et al*, 2008a,b). UNC119b was cloned in pET20 vector with a C-terminal histidine tag and purification was done by Nickel affinity purification followed by a size permeation chromatography. Site directed mutagenesis was performed by QuickChange kit from Stratagene.

Peptides

Fluorescently labelled N-terminal myristoylated peptides from GNAT1 (Myr-GAGASAEK-Fluorescein) and NPHP3 (Myr-GTASSLVSPA-

Fluorescein) were obtained from AltaBioscience. ODR3 (Myr-GSCQSNENSKG-OH) was prepared by standard Fmoc strategy. The N-myristoylated peptide (Myr-GSCQSNENSKG-OH) was then dissolved in DMSO, followed by the addition of excess fluorescein isothiocyanate and triethylamine. The product was further purified by HPLC to afford Myr-GSCQSNENSK(flourescein)G-OH.

In vitro fluorescence measurements

Fluorescence polarization measurements were done at 20°C in a buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂ and 2 mM βME. Data were recorded with a Fluoromax-2 spectrophotometer (S.A. Instruments, USA), with excitation and emission wavelengths at 490 and 520 nm for fluorescein-labelled peptided peptide and 366 and 450 for mant nucleotides. Data analysis was done with Graft 5.0 program (Erithracus software, Horley, UK).

The dissociation of a preformed 1 μM solution of a fluorescein-labelled myristoylated GNAT-1 peptide bound to UNC119a was started by injecting 100-fold excess unlabelled peptide or buffer in the presence and absence of 10 μM full-length Arl3•GppNHP, 10 μM full-length Arl2GppNHP or 10 μM truncated Arl2GppNHP. The reaction was followed by recording the polarization fluorescence in a stopped-flow apparatus (Applied Photophysics, Leaverhead, UK). The data were fitted to a single exponential function using Graft 5.0.

Crystallization and structure solution

Crystal trays of 12 mg ml⁻¹ of purified full-length Arl3•GppNHP in complex with UNC119a or ΔArl2(Q70L)•GppNHP in complex with UNC119a were set in the presence of 1:1000 (w/w) (trypsin) (Dong *et al*, 2007). Arl3GppNHP•UNC119a crystal complex appeared in Qiagen Classic suite and the condition was refined to 3.4 M Sodium Formate. Arl2GppNHP•UNC119a showed in Qiagen classic suite and the condition was refined to 2% PEG400 and 2M Ammonium Sulphate. The crystals were flash frozen in a buffer that contained the mother liquor components in addition to glycerol as a cryo-protectant (20 and 25%, respectively). Data collection was done at the X10SA beamline of the Swiss Light Source, Villigen. Data were processed by XDS (Kabsch, 1993). Molecular replacement was done using the program MolRep using 3GQQ (for UNC119a), 3BH6 (for Arl3) and 1KSH (for Arl2) as search models and refinement was done using Refmac 5 (Vagin and Teplyakov, 1997; Pannu *et al*, 1998). Ramachandran statistics for Arl3•GppNHP•UNC119a and ΔArl2(Q70L)•GppNHP•UNC119a showed 97.9 and 95.8% of all residues in the favoured region, respectively, and none were outliers. Atomic coordinates and structural factors were deposited at the Protein Data Bank (PDB) with accession codes, 4GOK and 4GOJ for ΔArl2(Q70L)•GppNHP•UNC119a and Arl3•GppNHP•UNC119a, respectively. For data collection and refinement statistics, see Supplementary Table 1. The morphing movie was created with the web server MovieMaker (<http://wishart.biology.ualberta.ca/moviemaker/>).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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