The Structure of Binder of Arl2 (BART) Reveals a Novel G Protein Binding Domain

IMPLICATIONS FOR FUNCTION

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The ADP-ribosylation factor-like (Arl) family of small G proteins are involved in the regulation of diverse cellular processes. Arl2 does not appear to be membrane localized and has been implicated as a regulator of microtubule dynamics. The downstream effector for Arl2, Binder of Arl2 (BART) has no known function but, together with Arl2, can enter mitochondria and bind the adenine nucleotide transporter. We have solved the solution structure of BART and show that it forms a novel fold composed of six \( \alpha \)-helices that form three interlocking “L” shapes. Analysis of the backbone dynamics reveals that the protein is highly anisotropic and that the loops between the central helices are dynamic. The regions involved in the binding of Arl2 were mapped onto the surface of BART and are found to localize to these loop regions. BART has faces of differing charge and structural elements, which may explain how it can interact with other proteins.

The Ras superfamily of small G proteins is composed of five families, with each family having diverse members and subfamilies. Small G proteins act as molecular signaling switches, cycling between an inactive, GDP bound conformation and an active, GTP bound conformation that is able to engage downstream signaling molecules and propagate signals from the membrane to the interior of the cell (reviewed in Ref. 1). Activation of these GT Pases is regulated by a class of proteins called guanine nucleotide exchange factors that catalyze the exchange of GDP for GTP in response to extracellular stimuli. Although small G proteins do possess intrinsic GTPase activity, the rate is too slow to be functionally applicable to cellular events. Rather, there is a family of GTPase activating proteins that increase the rate of intrinsic GTP hydrolysis (2). Structurally small G proteins share a common fold, the G domain, that includes two regions, known as switch 1 and switch 2, which are sensitive to the bound nucleotide and change conformation when GDP is exchanged for GTP.

The ADP-ribosylation factor (Arf) family of small G proteins are critical regulators of secretion, endocytosis, and phagocytosis (3). These proteins act at cellular and organelle membranes, e.g., Arf1, which recruits vesicle coat proteins to the Golgi (4) and Arf6, which activates lipid kinases at the plasma membrane (5). In common with other superfamily members, Arf proteins are post-translationally lipid modified to allow membrane localization. However, the Arf family is unusual in that this modification occurs at the N-terminal, conserved glycine 2, rather than at the C-terminal CAXX motif found in other small G proteins (reviewed in Ref. 6). Furthermore, the Arf proteins are unique in being myristoylated or acetylated, rather than farnesylated or geranylgeranylated. The conserved Gly residue is located within an N-terminal extension that, upon activation by GTP, forms an amphipathic helix. The lipid modification and amphipathic nature of this helix together serve to target and tether these proteins to cellular membranes (7).

One uniting structural feature of the Arf family is a mechanism that communicates the bound nucleotide status from the “front” of the molecule (the switch regions) to the “back” of the protein containing the N-terminal helix (reviewed in Ref. 8). In the GDP-bound conformation the N-terminal helix interacts with a hydrophobic binding pocket on the back surface of the protein. Upon nucleotide exchange and activation, two inter-switch \( \beta \)-strands, \( \beta2 \) and \( \beta3 \), undergo a 2-amino acid register shift relative to strand \( \beta1 \), deforming the N-terminal helix binding pocket and so displacing the helix, allowing it to interact with membranes. In this way, Arf proteins can efficiently couple GTP binding to membrane association.

The Arl (Arl-like) subfamily proteins share the same front-back signaling mechanism with the Arfs and are post-translationally modified on the conserved glycine 2 but although the Arfs share 40–70% homology to each other, the Arls are much more heterogeneous. Arls have been suggested to be involved in a diverse set of processes, including recruitment of GRIP (golgin-97, RanBP2a, 1m1h1p, and p230/golgin-245) domains to...
Golgi membranes (9, 10), trafficking processes in lysosomes (11), regulation of endosome-derived transport vesicles to the trans-Golgi network (3), regulation of release and uptake of prenylated proteins at the plasma membrane (12) and regulation of microtubule dynamics (13–15).

Arl2 is a 20.8-kDa cytosolic member of the Arl family whose distinguishing feature is that it is not modified at glycine 2 (16). Arl2 has been implicated as a regulator of microtubule dynamics or folding in a number of different assays and genetic screens (15, 17, 18), and these effects may be attributed to the interaction of Arl2-GDP with protein phosphatase 2A and Cofactor D. Arl2-GDP exerts an inhibitory influence on the microtubule destabilizing activities of Cofactor D, thereby acting as a microtubule stabilizing agent (19). This Arl2-Cofactor D complex has recently been shown to regulate the apical junction complex in epithelial cell monolayers, participating in the disassembly of the apical junction complexes and abrogating epithelial cell structure (20).

Binder of Arl2 (BART) was the first Arl2 effector protein to be isolated and identified (16). Arl2 and BART together enter the mitochondria and bind to the adenine nucleotide transporter (16), an inner mitochondrial transmembrane protein that is responsible for antiport of ADP/ATP and is also thought to be a component of the permeability transition pore that forms in response to apoptotic stimuli (21). More recently, BART has been shown to interact with and act as a nuclear retention factor for transcription factor STAT3 (22). However, the functional consequences of these interactions are presently unknown. BART also binds to Arl3, the closest homologue of Arl2, with whom it shares 53% sequence identity and a high degree of structural conservation (23). Like Arl2, Arl3 has been shown to regulate microtubule-dependent processes (15). Arl2, Arl3, and BART associated with centrosomes at all stages of the cell cycle where they may play a role in the growth and regulation of microtubules and the mitotic spindle (15).

Here we present the solution structure and dynamics of BART solved by NMR spectroscopy, and reveal it to be composed entirely of α-helices, arranged in a novel topology. We show that BART binds to Arl2-GTP with nanomolar affinity and use chemical shift mapping to reveal the binding site for Arl2-GMPPNP on the surface of BART.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The expression construct for full-length Arl2 (Arl2) (accession number Q9D0J4) in pGex-KG was a kind gift from Prof. A. Wittinghofer. Full-length BART-(1–163) (FL BART) (accession number Q9Y2Y0), BART-(1–136) (BART), and BART-(14–136) were cloned into the BamHI and BglII sites of pET-16b (Novagen).

**Protein Expression**—His10 fusion proteins were expressed in Escherichia coli BL21(DE3) cells. Stationary overnight cultures were diluted 1 in 10 and grown at 37 °C until an A600 0.7 was reached, then induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h. The fusion protein was affinity purified using a Ni-IDA column (Novagen). His10-tagged BART for the scintillation proximity assay was applied directly to a 16/60 S75 column (GE Healthcare) while protein to be used for structural studies was incubated with Factor Xa (Roche) to cleave the tag prior to gel filtration. To produce isotopically labeled protein for NMR studies, cells were grown in MOPS supplemented with 5% Cellebrite (Spectra Stable Isotopes) with 15NH4Cl and appropriately labeled glucose and purified as before. Protein concentrations for all proteins were evaluated by the Bio-Rad Protein Assay (Bio-Rad) and compared with a standard curve. GST-tagged recombinant proteins were expressed in E. coli BL21 cells. Stationary overnight cultures were diluted 1 in 10 and grown at 37 °C until an A600 0.7 was reached, cooled to 20 °C, and induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 16 h. GST-tagged protein was affinity purified using glutathione-agarose beads (Sigma), and eluted from the beads by cleavage of the GST tag by thrombin (Merck) prior to gel filtration on a 16/60 S75 column (GE Healthcare). Protein concentrations for all proteins were evaluated as before.

**Nucleotide Exchange**—For NMR titrations with BART, Arl2 was concentrated and the bound nucleotide exchanged for the non-hydrolysable GTP analogue GMPPNP (Sigma) as described previously (24). For use in scintillation proximity assays a [3H]GTP complex of Arl2 was made. [8,5-3H]GTP (0.15 mCi, GE Healthcare) was dried by centrifugal evaporation. 0.7 mg of Arl2 was added (in 145 μl), followed by 16 μl of 3 M (NH4)2SO4. The mixture was incubated at 30 °C for 1 h after which 1 μl of 1 M MgCl2 was added. Unbound nucleotide was removed using a 1-ml pre-spun G-25 Sephadex (Superfine, GE Healthcare) column in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol.

**Scintillation Proximity Assays (SPAs)**—The affinity of Arl2 protein for His10-BART proteins was measured using SPAs in which the His10-BART was attached to a fluoromicrosphere via an anti-His antibody in the presence of Arl2[3H]GTP. Binding of Arl2-[3H]GTP to His10-BART brings the labeled nucleotide close enough to the scintillant to obtain a signal. An apparent Kd value for the interaction was measured as described previously (25).

**NMR Spectroscopy of BART**—All NMR experiments were run at 298 K. BART was concentrated to ~1 mM in NMR buffer (20 mM sodium phosphate, pH 6, 150 mM NaCl, 10% D2O, 0.05% NaN3). 15N-HSQC, 15N-separated NOESY (100 ms mixing time), and 15N-separated TOCSY (43 ms mixing time) were recorded on Bruker DRX500, 13C-HSQC, 13C-separated NOESY (100 ms mixing time), and 13C-separated HCCH-TOCSY (480-16 mixing, 18 ms mixing time), intra-HNCA, HN(CO)CA, HN(CD)CA, and HN(CD)CAB were recorded on a Bruker DRX600. Backbone torsion angles were estimated from CA, CO, CB, N, and HA chemical shifts using the program TALOS (26). NMR data were processed using the AZARA package and analyzed using ANALYSIS (27).

**Backbone Dynamics**—Two-dimensional 1H-15N correlation spectra (28) were recorded on 15N-labeled BART on a Bruker DRX600 at 298 K, to determine 15N relaxation times and the 1H-15N NOE. The 15N T1 and T2 series of spectra were recorded as pseudo three-dimensional experiments. T1 experiments were recorded with time delays of 0.01, 0.05, 0.10, 0.15, 0.25, 0.40, 0.50, 0.65, 0.80, and 1.00 s and the T2 series were recorded with delays of 0.0144, 0.0288, 0.0432, 0.0576, 0.0720, 0.0864, 0.1008, 0.1152, 0.144, and 0.173 s. The relaxation data were
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analyzed using CCPN Analysis (27), fitting a relaxation time and associated error for each residue (30). \( T_1 \) and \( T_2 \) were fitted to an exponential: \( I(t) = I_0 \exp(-t/T_{1,2}) \). Steady-state NOE data comprised a single pair of reference and saturated experiments. The peak heights were measured and the intensity ratios \( I_{sat}/I_{ref} \) were calculated. The error in the NOE, \( \delta \text{(NOE)} \), was estimated:

\[
\delta \text{(NOE)} = \text{NOE} \times \sqrt{\left(\frac{\delta I_{sat}}{I_{sat}}\right)^2 + \left(\frac{\delta I_{ref}}{I_{ref}}\right)^2}
\]  

where \( \delta I_{sat} \) and \( \delta I_{ref} \) are the noise levels in the saturated and reference experiments, respectively.

The relaxation data were analyzed using the program Tensor2 (31), based on the residue-specific \( R_1 \) and \( R_2 \) relaxation rates, the heteronuclear NOE values, and the coordinates of the BART structure calculated without diffusion anisotropy refinement.

The average \( R_2/R_1 \) was calculated using relaxation data for those residues in secondary structure that were deemed not to have significant internal motion, *i.e.* those with heteronuclear NOE values more than 0.65. The average \( R_2/R_1 \) was calculated for residues 21–33, 35–51, 61–81, 83, 90–96, 98–101, 106, 111–116, 118–120, and 122–133 and was used to obtain an estimate for the overall correlation time, \( \tau_c \), and the principal components of the rotational diffusion tensor. Order parameters (\( S^2 \)) were calculated for all residues for which data were available, using an N-H bond length of 1.02 Å. Data were analyzed using the Lipari-Szabo model-free formalism (32, 33) or the extended model (34).

**Structure Calculation**—Structures of BART-(1–136) were calculated iteratively using CNS 1.0 interfaced to Aria 1.2 (35). The \( \phi \) and \( \psi \) restraints from TALOS were included with an error of ±30%. Structure calculations were also performed using diffusion anisotropy restraints to refine the orientation of the backbone amide bond vectors (36) using the extended version of ARIA 1.2 (37). An initial estimate of the anisotropy (\( A \)) and rhombicity (\( \eta \)) was obtained based on the unrefined structure and the restricted set of \( T_1/T_2 \) ratios described above using Tensor2. A grid search was then used to optimize the values of \( A \) and \( \eta \) during the ARIA run as described previously (37).

**NMR Titration**—\(^{15}N\) BART-(14–136) was exchanged into Arl2 NMR buffer (50 mm sodium phosphate, pH 6.5, 250 mm NaCl, 5 mm MgCl\(_2\), 10% D\(_2\)O, 0.05% NaN\(_3\)), using a PD10 column (Sigma). \(^{15}N\)-HSQC experiments were recorded at BART-(14–136):Ar2GMPNNP ratios of 1:0, 1:0.75, and 1:1.2. The chemical shift changes, \( \delta \), for residues whose resonances could be tracked were calculated using the equation,

\[
\delta = \sqrt{\delta_{1H}^2 + (0.15\delta_{15N})^2}
\]

where \( \delta_{1H} \) and \( \delta_{15N} \) are the chemical shift changes for the \(^{1}H\) and \(^{15}N\) dimensions, respectively. Residues that had shifted too far to be reliably tracked were assigned a \( \delta \) value of 0.3 and residues that were too overlapped to be reliably assigned in the complex spectra were assigned a \( \delta \) value of 0.

The residues that had shifted significantly (\( \delta > 0.1 \)) were filtered to remove amides in residues that were not solvent exposed in the structure.

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**TABLE 1**

| Experimental restraints and structural statistics | Number of experimental restraints |
|---|---|
| Unambiguous | 2185 |
| Ambiguous | 432 |
| Dihedral restraints from Talos | 99 |
| Coordinate precision | (SA)\(^a\) |
| R.m.s. deviations of backbone atoms (16–133) (Å) | 0.48 ± 0.01 |
| R.m.s. deviations of all heavy atoms (18–133) (Å) | 1.01 ± 0.07 |
| R.m.s. deviations | (SA)\(^b\) |
| From the experimental restraints NOE distances (Å) | 0.46 ± 0.005 |
| Talos dihedral angles (Å) | 0.0011 ± 0.00005 |
| From idealized geometry | 0.0015 |
| Bonds (Å) | 0.28 ± 0.0043 |
| Angles (°) | 0.16 ± 0.009 |
| Improper (°) | 0.28 |

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\( ^a \text{(SA)} \) represents the average r.m.s. deviations for the ensemble.

\( ^b \text{(SA)} \), represents values for the structure that is closest to the mean.

\( ^c \) The Lennard-Jones potential was not used at any stage in the refinement.

**RESULTS**

**BART Structure**—The BART construct used in NMR samples for structure calculation was truncated C-terminal to residue 136, as initial investigations with full-length protein suggested that the C-terminal 28 amino acids were prone to degradation during the purification. The backbone resonances were assigned using three-dimensional i-HNCA (38), HN(CO)CA, HNCACB, and HN(CO)CAB spectra (reviewed in Ref. 39) on a \(^{15}N^{15}C\)-labeled protein sample analyzed with reference to a two-dimensional \(^{1}H^{15}N\)-HSQC experiment. No backbone resonances were observed for residues 1–13, and subsequent N-terminal sequencing (Edman) showed that the N terminus of the protein was being proteolytically cleaved and degraded (data not shown). A new clone comprising residues 14–136 was subsequently constructed and the N terminus found to be stable. Side chain assignments were obtained from three-dimensional HCCH-TOCSY and \(^{15}N\)-separated TOCSY spectra. NOE restraints were collected from three-dimensional \(^{15}N\)-separated NOESY (907 NOEs) and \(^{13}C\)-separated NOESY (2,236 experiments).

Initial structures were calculated using a total of 2,279 unique (non-degenerate) NOE restraints (1,687 unambiguous and 712 ambiguous). After 8 iterations, there were 2,093 unambiguous and 186 ambiguous NOEs. In the final iteration, 100 structures were calculated; the 50 with the lowest energy were selected for analysis.

The structure of BART-(14–136) is well defined by the NMR data and has good covalent geometry (Table 1). BART is composed of 6 \( \alpha \)-helices, labeled H1–H6 (Fig. 1) joined by short loops (L1–L5). H1 comprises residues 19–32 and is closely followed by H2 (residues 35–51); the relative orientation of these helices is ~135° and together they resemble a continuous \( \alpha \)-helix with a central elbow. H3 (residues 62–82) is approximately...
parallel to H2 and is followed by H4 (residues 90–100), which is at –90° to H3. H5 (residues 106–116) is approximately parallel to H2 and H3 and is closely followed by H6 (residues 118–132), at –60° to H5; these final two α-helices also resemble a continuous helix with a central elbow.

Overall, the structure of BART comprises three interlocking L-shapes, where the long arm of the first two Ls (helix H2 and H4) and the short arm of the third L (helix H5) form a three-helix bundle at the center of the structure. This bundle is capped and reinforced by the other three helices, H6 at the top (Fig. 1) and H1 and H4 at the bottom. This arrangement of α-helices in BART appears to be a novel topology (Fig. 1) and no structural homologues to BART were found when it was compared with the contents of the PDB using the Dali server (40).

The interesting topology of BART can be attributed to the high abundance of aromatic groups (19/116 structured residues). These bulky side chains both pack the helices together and orientate them. Phe-21 and Val-24 are responsible for packing H1 of BART against H4, causing H4 to curve outwards by packing with Thr-96, Leu-97, His-100, and Val-104 (Fig. 2A). Aliphatic side chain interactions consolidate the packing between helices H1, H2, and H3, preventing the formation of a single, extended helix by H1 and H2 (Fig. 2B). The positioning of H5 and H6 against H2 and H3 arises mostly through interaction of aromatic side chains. Leu-112 and Phe-115, at the C terminus of H5, pack against Ile-77, Glu-74, and Ile-70 on H3 and lock H5 into place against H3 (Fig. 2C). Asp-117, in the short loop between H5 and H6, is solvent exposed and orientated away from these neutral regions, allowing H6 to swing around almost 90° relative to H5. Pivotal to H6 positioning against H2 and H3 are packing interactions involving the aromatic side chains of Tyr-128, Phe-125, Phe-121, and Phe-118 of H6; Tyr-48, Tyr-49, and Phe-52 of H2; and Tyr-62 and Phe-66 of H3 (Fig. 2D).

**Backbone 15N Dynamics—** Experimental relaxation data consisting of 15N longitudinal relaxation times ($T_1$), transverse relaxation times ($T_2$), and the steady-state 1H-15N-heteronuclear NOE were obtained for all residues whose backbone amide resonances were assigned, unless they were overlapped in the 15N-HSQC spectra or the fits of the intensities did not follow a single exponential decay. Relaxation parameters were available for a total of 91 residues. Plots of $T_1$, $T_2$, the NOE, and the $T_1/T_2$ ratio are shown in Fig. 3. The average values for $T_1$, $T_2$, and the NOE are 725.41 ± 42.9, 91.15 ± 14.21, and 0.78 ± 0.09 ms, respectively.

Residues that do not experience significant internal motion and that are within the secondary structure were used to esti-
provide validation of the orientation of the helices. The orientations of the helices are well fixed by the hydrophobic core, suggesting that the helical bundle is not undergoing any large-scale concerted motions and that it tumble anisotropically in solution, with principal anisotropy values of 1.37 and 0.505 for A and \( \eta \), respectively. These were then optimized by a grid search (37) during structural refinement against the \( T_1/T_2 \) values (36). The refinement against the \( T_1/T_2 \) values led to a slight reorientation of the helices and a small improvement in the Ramachandran plot statistics but overall the structures of the unrefined and refined BART are very similar, with an r.m.s. deviation over all backbone atoms of 1.4 Å.

**Analysis of the Interaction between Arl2 and BART**—Scintillation proximity assays were used to calculate the affinity of the interaction between the different BART proteins and Arl2 (Fig. 5) to ensure that removal of the N and C termini of BART did not affect the binding to G proteins. The \( K_d \) for full-length BART, BART-(1–136), and BART-(14–136) were 40, 41, and 34 nM, respectively, indicating that neither the extreme N terminus nor the C terminus is required for the interaction with Arl2. These affinities are in agreement with the previously published \( K_d \) of the interaction (41, 42) and is similar to the affinities we have observed for other small G protein-effector complexes (25, 43).

The binding site of Arl2 on BART was mapped by titrating unlabeled Arl2-GMPPNP into \(^{15}N\)-labeled BART-(14–136) and recording \(^{15}N\)-HSQC spectra to monitor the changes (Fig. 6A). If a backbone amide in BART is in the vicinity of the binding interface, its chemical environment will change when Arl2 and BART interact, giving rise to a change in the chemical shift of the amide in the \(^{15}N\)-HSQC. In the BART-Arl2 spectra, all of the resonances whose shifts change significantly are in slow exchange. There is no gradual shift as Arl2 is added but rather the spectra are characterized by the appearance of new resonances as the ratio of protein concentrations approaches 1:1, with a concomitant disappearance of the free protein resonances. The resonances of the complex are also decreased in intensity due to the increase in the correlation time when the 13.5-kDa BART is incorporated into the 35-kDa BART-Arl2 complex. The resonances that had completely disappeared from the complex spectrum were assumed to be those whose chemical environment had changed in the complex. NMR mapping often overestimates the residues involved in protein binding surfaces due to secondary effects, such as slight reorientations of the structural elements, leading to shifts in resonances in residues outside the true interface. The overall chemical shift change, \( \delta \), of each backbone amide resonance that could be tracked was calculated. Residues with \( \delta > 0.1 \) were deemed have to have shifted significantly. To reduce inclusion of amides that were not directly in contact with Arl2, the solvent accessibility of BART was calculated using NACCESS (47). The backbone amides for residues that were not solvent exposed (less than 50%) were excluded. The changes in BART upon Arl2-GMPPNP binding were found to be localized to four

![Relaxation data for BART](image_url)

**FIGURE 3.** Relaxation data for BART. A, the \(^{1}H\) \( T_1 \), B, the \(^{15}N\) \( T_1 \), C, the heteronuclear NOE. The positions of the \( \alpha \)-helices are marked by black cylinders at the top of the figure. The horizontal black lines in A–C show the average value for that parameter.
regions of the protein, three of which encompass the longer loops that we have shown to be more dynamic (Fig. 6B): region 1, the C terminus of helix H1, the loop L1, and the N terminus of helix H2 (Asp-30, Met-33, Asp-35, and Gln-38); region 2, loop L2 (Thr-55 and Asn-58); region 3, loop L3 (Gln-83, Gly-87, and Asn-89); and region 4, the C terminus of helix H4 and loop L4 (His-99 and Asp-102).

DISCUSSION

The solution structure of BART as solved by NMR spectroscopy reveals the protein to comprise entirely α-helical secondary structure elements. These helices are packed together in a novel manner, such that no structural homologues of BART are found in the Protein Data Bank. Analysis of the NMR relaxation experiments show three loops to be more flexible than the rest of the protein (L2, L3, and L4), whereas analysis of the chemical shift mapping data of Arl2 onto BART shows that a defined region, comprising loops L1–L4 and parts of helix H2, is involved in the binding reaction. The apparent $K_d$ of the BART proteins for Arl2-GTP calculated by SPA were all in the low nanomolar range, which is in excellent agreement with previously published $K_d$ values of the interaction (41).

As discussed, there are no structural homologues of BART in the Protein Data Bank. A Blast search, however, revealed a sequence homologue in the Protein Data Bank, the Arl2 binding protein from zebrafish (PDB code 2K0S). The zebrafish protein is also composed of 6 α-helices, encompassing approximately the same residue ranges as those in human BART. The sequence identity of the two proteins over the structured regions (residues 14–136) is 74%. The overall topology of the two structures is, however, quite different. We used the $T_1/T_2$ values that were measured on human BART and attempted to use them to calculate the rotational diffusion tensor of the zebrafish protein using Tensor2. The rotational diffusion tensor could not be fitted with any model (axially symmetric or fully asymmetric) using the zebrafish structure, based on the $x^2$ test. The $T_1/T_2$ ratios calculated by Tensor2 versus those observed are shown in Fig. 4B. Comparison of these with the $T_1/T_2$ ratios calculated for the BART structure that had not been refined with diffusion anisotropy restraints (Fig. 4A) shows that the human BART structure fits the $T_1/T_2$ data much better than the zebrafish protein. The zebrafish Arl2-binding protein structure is of a lower resolution, which may account for some of the differences we observe.

Canonical small G protein effectors typically make contacts with the switch 1 and switch 2 regions of the small G protein, as these are the regions that undergo a large conformational change upon activation of the protein. In several small G pro-
tein-effector complexes, for example, Ras-effector complexes (44), Cdc42-effector interactions (45) (24), and the Arl2-phosphodiesterase δ interaction (46), an intermolecular β-sheet is observed, formed by the interaction between the β2 strand of the G protein and a β-strand provided by the effector. In the case of the BART-Arl2 complex, this is clearly not the case as BART contains no β-sheet, nor any extended unstructured regions that could form a β-sheet upon binding. Although α-helical regions of large, multidomain proteins interact with small G proteins, such as the interaction between the homology region 1 domains of protein kinase C-related kinase (PRK1) with RhoA and Rac1 (43), these are often discrete coiled-coil regions of the effector proteins that contact residues near the switch regions. Of the α-helical effectors whose interactions with small G proteins are known, the majority of them interact with the switch 2 α-helix, in a parallel or anti-parallel fashion.

The residues of BART involved in binding Arl2 are mainly found in flexible loop regions L2–L4 and helices H1 and H2 and the “elbow” between them. Most resonances for the residues in these loop regions were too overlapped to accurately track changes during the course of the titration, so it is highly likely that there are more residues involved in binding Arl2. The solvent-exposed residues that shift on Arl2-binding map to the same face of BART, with the exception of Thr-55 and Asn-58. There were, however, shifts observed for resonances in all of the α-helices that were removed because they are not accessible to the solvent. The large number of shift changes suggests that there is a subtle reorientation of the helices when BART binds to Arl2. This is not unreasonable in a completely solvated structure as the residues that undergo a large structural reorganization upon nucleotide exchange. The switch regions map to the same face of Arl2, making it unlikely that the Arl2 binding site on BART will involve regions of BART that are far apart in the structure. The structure of BART is sufficiently different to that of other small G protein effector molecules that prediction of its mode of binding by comparison to these proteins is not possible. The switch regions of small G proteins are known to be flexible, so it is tempting to suggest that the ARl2-BART interaction involves the union of the flexible switches with the dynamic loops of BART and that the resulting rigidity of the loops leads to the minor shifting of the BART helices. Confirmation of this awaits the determination of the high-resolution structure of the complex.

BART has previously been shown to be an Arl3 effector (16), and Arl3 utilizes the same binding sites of BART as does Arl2 (data not shown). However, it has been demonstrated that BART can also interact with other proteins, namely the adenine nucleotide transporter isoform 1 (16) and STAT3 (22). What is surprising is that BART can bind to these two proteins both independently and as a BART-Arl2 complex, implying that the surfaces of BART utilized by adenine nucleotide transporter isoform 1 and STAT3 to bind are distinct from those that bind Arl2. This is not an unreasonable assumption when the overall shape of BART is considered, as it displays distinct surfaces of mixed charge and structural elements. Arl2 is the only member of the Arf and Arf-like protein family not to be post-translationally modified at the highly conserved residue Gly-2 (16), and so it is tempting to speculate that BART could be acting to tether Arl2 to the mitochondrial membrane via its interaction with adenine nucleotide transporter isoform 1 and so be acting as an adaptor protein in this context. The discovery that ELMOD2, the only Arl2 specific GAP found to date, is found as part of a
large membrane-associated complex and whose activity is strongly dependent on the presence of lipids (29) lends weight to the idea that Arl2 may be regulated at membranes. This would create a requirement for an otherwise soluble, cytosolic protein to be membrane associated in the absence of lipid modifications and BART may play a role in fulfilling this requirement.

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REFERENCES

1. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) Nature 348, 125–132
2. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127
3. Burd, C. G., Strochlic, T. I., and Gangi Setty, S. R. (2004) Biochimie 86, 4675–4684
4. Kawasaki, M., Nakayama, K., and Wakatsuki, S. (2005) Trends Cell Biol. 15, 681–689
5. Nie, Z., Hirsch, D. S., and Randazzo, P. A. (2006) Curr. Opin. Cell Biol. 18, 125–132
6. Casey, P. J. (1994) Curr. Opin. Cell Biol. 6, 219–225
7. Linge, J. P., O’Donoghue, S. I., and Nilges, M. (2001) Biochem. Biophys. Res. Commun. 289–302
8. Pasqualato, S., Renault, L., and Cherfils, J. (2002) Curr. Opin. Cell Biol. 14, 579–591
9. Mott, H. R., Owen, D., Nietlispach, D., Lowe, P. N., Manser, E., Lim, L., and Boelens, R. (2004) J. Mol. Biol. 340, 167–176
10. Jackson, C. (2003) Mol. Biol. Cell. 14, 2476–2487
11. Bhamidipati, A., Lewis, S. A., and Cowan, N. J. (2000) J. Cell Biol. 149, 1087–1096
12. Radcliff, P., Vardy, L., and Toda, T. (2000) FEBS Lett. 468, 84–88
13. Shultz, T., Shmuel, M., Hyman, T., and Altschuler, Y. (2008) FEBS J. 22, 168–182
14. Belzacq, A. S., Vieira, H. L., Kroemer, G., and Brenner, C. (2002) Biochimie (Paris) 84, 167–176
15. Muramoto, R., Sekine, Y., Imoto, S., Ikeda, O., Okayama, T., Sato, N., and Matsuda, T. (2008) Int. Immunol. 20, 395–403
16. Hillig, R., Hanzal-Bayer, M., Linari, M., Becker, J., Wittinghofer, A., and Renault, L. (2000) Structure 8, 1239–1245
17. Antoshechkin, I., and Han, M. (2002) J. Biol. Chem. 277, 10636–10643
18. Shern, J. F., Shmuel, M., Hyman, T., and Altschuler, Y. (2008) Mol. Biol. Cell. 19, 910–920
19. Shern, J. F., Shmuel, M., Hyman, T., and Altschuler, Y. (2008) J. Biol. Chem. 283, 1692–1704
20. Belzacq, A. S., Vieira, H. L., Kroemer, G., and Brenner, C. (2002) Biochimie (Paris) 84, 167–176
21. Muramoto, R., Sekine, Y., Imoto, S., Ikeda, O., Okayama, T., Sato, N., and Matsuda, T. (2008) Int. Immunol. 20, 395–403
22. Hillig, R., Hanzal-Bayer, M., Linari, M., Becker, J., Wittinghofer, A., and Renault, L. (2000) Structure 8, 1239–1245
23. Owen, D., Mott, H. R., Laue, E. D., and Lowe, P. N. (2000) Biochemistry 39, 1243–1250
24. Owen, D., Campbell, L., Littlefield, K., Etverts, K., Li, Z., Sacks, D., Lowe, P., and Mott, H. (2008) J. Biol. Chem. 283, 1692–1704
25. Sharer, J. D., and Kahn, R. A. (1999) J. Biol. Chem. 274, 289–302
26. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, M., Ulrich, E. L., Markley, J. L., Ionides, J., and Laue, E. D. (2005) Proteins 59, 687–696
27. Farrow, N. A., Zhang, O., Forman-Kay, J. D., and Kay, L. E. (1994) J. Biol. Chem. 269, 11903–11906
28. Farrow, N. A., Zhang, O., Forman-Kay, J. D., and Kay, L. E. (1994) J. Biol. Chem. 269, 11903–11906
29. Bowzard, J. B., Cheng, D., Peng, J., and Kahn, R. A. (2007) J. Biol. Chem. 282, 17568–17580
30. Effron, B., and Tibshirani, R. (1986) Stat. Sci. 1, 54–77
31. Litvinov, I., and Wang, S. (2006) Biochemistry 45, 727–734
32. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4546–4559
33. Lipari, G., and Szabo, A. (1982) J. Am. Chem. Soc. 104, 4546–4559
34. Clore, G. M., Szabo, A., Bax, A., Kay, L. E., Driscoll, P. C., and Gronenborn, A. M. (1990) J. Am. Chem. Soc. 112, 4989–4991
35. Linge, J. P., O’Donoghue, S. I., and Nilges, M. (2001) Methods Enzymol. 319, 71–90
36. Tjandra, N., Farrow, N. A., and Bax, A. (1995) J. Am. Chem. Soc. 117, 12562–12566
37. Houben, K., Dominguez, C., van Schaik, F. M., Timmers, H. T., Condon, J. E., and Boelens, R. (2004) J. Mol. Biol. 340, 513–526
38. Nietlispach, D., Ito, Y., and Laue, E. D. (2002) J. Am. Chem. Soc. 124, 11199–11207
39. Ferentz, A., and Wagner, G. (2000) Q. Rev. Biophys. 33, 29–65
40. Holm, L., and Sander, C. (1996) Science 273, 595–603
41. Sharer, J. D., and Kahn, R. A. (1999) J. Biol. Chem. 274, 27553–27561
42. Veltel, S., Kravchenko, A., Ismail, S., and Wittinghofer, A. (2008) FEBS Lett. 582, 2501–2507
43. Owen, D., Lowe, P. N., Nietlispach, D., Brosnan, C. E., Chirgadze, D. Y., Parker, P. J., Blundell, T. L., and Mott, H. R. (2003) J. Biol. Chem. 278, 50578–50587
44. Nossal, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) Nature 375, 554–560
45. Houben, K., Dominguez, C., van Schaik, F. M., Timmers, H. T., Condon, J. E., and Boelens, R. (2004) J. Mol. Biol. 340, 513–526
46. Nietlispach, D., Ito, Y., and Laue, E. D. (2002) J. Am. Chem. Soc. 124, 11199–11207
47. Hubbard, S. J., and Thornton, J. M. (1993) NACCESS, University College, London