The ARF-like 2 (ARL2)-binding Protein, BART

PURIFICATION, CLONING, AND INITIAL CHARACTERIZATION*

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ARF-like proteins (ARLs) comprise a functionally distinct group of incompletely characterized members in the ARF family of RAS-related GTPases. We took advantage of the GTP binding characteristics of human ARL2 to develop a specific, high affinity binding assay that allowed the purification of a novel ARL2-binding protein. A 19-kDa protein (BART, Binder of ARL Two) was identified and purified from bovine brain homogenate. BART binding is specific to ARL2-GTP with high affinity but does not interact with ARL2-GDP or activated ARF or RHO proteins. Based on peptide sequences of purified bovine BART, the human cDNA sequence was determined. The 489-base pair BART open reading frame encodes a novel 163-amino acid protein with a predicted molecular mass of 18,822 Da. Recombinant BART was found to bind ARL2-GTP in a manner indistinguishable from native BART. Northern and Western analyses indicated BART is expressed in all tissues sampled. The lack of detectable membrane association of ARL2 or BART upon activation of ARL2 is suggestive of actions quite distinct from those of the ARFs. The lack of ARL2 GTPase-activating protein activity in BART led us to conclude that the specific interaction with ARL2-GTP is most consistent with BART being the first identified ARL2-specific effector.

The ADP-ribosylation factor (ARF) family (1–4) of Ras-like GTPases can be divided into two distinct groups based on both structural and functional considerations (14). The ARF proteins, numbering at least six distinct genes/proteins in mammals, are at least 60% identical at the amino acid level, and all share certain activities, specifically complementation of the lethal yeast arf1–arf2 knockout mutant strain, co-factor activity in the bacterial toxin-catalyzed ADP-ribosylation of Gα, and activation of phospholipase D (15–17). The ARF family also includes a group of more structurally divergent ARF-like proteins (ARLs) with 40–60% sequence identity to any ARF or to each other. The ARLs lack each of these ARF activities, although reports of relatively low activity have emerged for human ARL1 (18). Only one ARL, so far, has been shown to be essential; deletion of ARF-like 1 in Drosophila melanogaster is lethal at an early embryonic stage (19). Each ARL sequenced to date has a glycine at position 2, the site of myristoylation on ARFs. Yeast ARL1 (20) and human ARL2 have been shown to be N-myristoylated by co-expressing each in bacteria with an N-myristoyltransferase. There is a possibility that human ARL2 and ARL3 are not acylated, as they each fail to incorporate radioiodelabel myristate when co-expressed with the N-myristoyltransferase (21). A lack of acylation would likely indicate a difference in cellular mechanisms as N-myristoylation of ARFs is essential in yeast and critical to a number of biochemical activities found for ARF proteins.

ARFs offer a good example of diversity in GTPase signaling, as ARF proteins can regulate mitotic growth (5), sporulation (6), respiration (5), and vesicular membrane traffic (7, 8) in the yeast, Sacccharomyces cerevisiae, despite the presence of only two ARF genes/proteins. ARF exchange factors (9, 10) and GAPs (11–13) have been described, and each possess features in their primary sequences that may help define them, the SEC7 and ARF GAP domains, respectively. In contrast, with the exception of ARF GAPs, each of the ARF effectors identified to date lack common structural motifs or features (5).

Although there exists at least a partial understanding of a cellular role for ARF proteins, little is known about the function of any of the ARL proteins. To begin to define one or more signaling pathways for ARL proteins in mammals, we attempted to identify and characterize specific ARL-binding proteins. The ability of ARL2 to bind activating guanine nucleotide triphosphates to high stoichiometry, in the absence of detergents or lipids (22), allowed the use of techniques not possible with ARF proteins. One such approach is the GTPase overlay, used successfully in previous studies to identify factors that interact with other small GTPases, such as RAC or CDC42 (23, 24) and Ran (25). In this report we describe the identification, purification, and initial characterization of a novel 19-kDa bovine brain protein, BART, which specifically interacts with ARL2-GTP. This is the first protein shown to interact with an ARL protein. Based on the specificity and affinity of BART binding of ARL2-GTP, we conclude that BART is an effector for ARL2.

MATERIALS AND METHODS

Gel Electroforesis and Immunoblotting—Protein samples were prepared in Laemmli’s sample buffer (26) and boiled for 5 min before loading onto 12.5% or 15% polyacrylamide gels. Resolved proteins were either stained with colloidal brilliant blue (Sigma) or transferred elec...
trophoretically to nitrocellulose filters (Bio-Rad), for 2 h at 60 V in Towbin’s buffer (50 mM Tris base, 0.38 mM glycine, 0.1% SDS, 20% methanol (27)). Immunoblots were performed as described previously (28) using horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Filters were developed for enhanced chemiluminescence (Amersham Pharmacia Biotech, ECL) to visualize reactive proteins.

**GTPase Overlay Assay**—The GTPase overlay assay was developed by modifying the procedure of Lounsbury et al. (25). Up to 25 μg of protein was loaded per lane and resolved and transferred to nitrocellulose filters, as described above for immunoblots. Filters were then incubated for at least 1 h at 4 °C in renaturation buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM MgOAc, 0.25% Tween 20, 5 mM dithiothreitol (DTT), and 0.5% bovine serum albumin (BSA)). The filter was then equilibrated by shaking in 15-mL binding buffer (20 mM MOPS pH 7.1, 100 mM KOAc, 5 mM MgOAc, 0.1% Triton X-100, 0.5% BSA, 5 mM DTT, 50 μM GTP, and 50 μM GDP) for at least 30 min at room temperature (RT). Radioactive ARL2-GTP was produced by incubating 2 μg of purified recombinant ARL2 with 20 μCi of [α-32P]GTP (3,000 Ci/mmol; NEN Life Science Products) for 15 min at 30 °C in 15 μL of loading buffer, consisting of 20 mM MOPS, pH 7.1, 1 mM EDTA, 0.05 mM MgOAc, 1 mM BSA, and 1 μM GDP. Approximately 70% of the radiolabeled nucleotide is bound to protein at the end of this loading reaction. The filter was then incubated with the ARL2-[α-32P]GTP mixture at 15 °C with gentle shaking. Note that the binding buffer (also used for washes) contains 50 μM GTP and GDP to lower the specific activity of free nucleotides to such a point that binding of radiolabeled nucleotides by filter-bound nucleotide-binding proteins is insignificant. The filter was then washed 3 times with binding buffer and then exposed to x-ray film (Eastman Kodak Co., XAR) or phosphorimages (Molecular Dynamics Storm PhosphorImager) for analysis.

**Protein Purification**—All resins were obtained from Amersham Pharmacia Biotech, except for Ultrogel AcA54 (Bio Sepra). All purification procedures were carried out at 4 °C unless otherwise indicated. Frozen bovine brains (~300 g; Pel-Freeze) were homogenized with a Polytron in 300 mL of ice-cold buffer, consisting of 20 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT (TNMD), and protease inhibitors (40 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 0.5 μg/mL aprotinin). The resulting homogenate was filtered through cheese cloth and spun at 100,000 × g for 60 min at 4 °C. The supernatant (S100) was then applied to a 1 liter (bed volume) DEAE-Sephacel column (50 mm × 60 cm) and developed with a 0.1–1.0 mM linear NaCl gradient in TNMD buffer at 240 mL/h. Fractions (15 mL) were assayed by gel electrophoresis, and fractions containing the desired activity were pooled. The column range were pooled. ARL2-GTP binding activity eluted as a single peak, at a position corresponding to approximately 420 mM NaCl. The DEAE pool was slowly brought to 2 M ammonium sulfate at 4 °C with stirring for 30 min and then clarified by centrifugation at 10,000 × g for 30 min. The resulting pellet was resuspended in a minimal volume of TNMD and applied to an AcA54 column (1-liter bed volume; 50 mm × 60 cm) at a total flow rate of 1 mL/min. Fractions containing ARL2 were pooled and further purified by chromatography on a Superdex 75 gel filtration column (Amersham Pharmacia Biotech), according to manufacturer’s instructions. Eluted BART-6His was further purified, and excess metal ions were removed by chromatography on a Superdex 75 gel filtration column (24 mL; 10 mm × 30 cm, Amersham Pharmacia Biotech). Recombinant proteins ARL2 was purified as described (14). All protein concentrations were determined using the Bradford assay (Bio-Rad) (30).

**Mant-Gpp(NH)p Fluorescence**—To assay binding between BART and activated ARL2 in solution, changes in the fluorescence of ARL2-bound N-methylanthraniloyl (Mant)-Gpp(NH)p were monitored. Recombinant ARL2-GTP was preincubated in a binding buffer (20 mM Hepes, pH 7.4, 2 mM EDTA, 100 mM NaCl, 2 mM DTT, 1 mM MgCl2, 0.1 mg/mL BSA (HENDM) at RT, until fluorescence reached equilibrium (typically 5–10 min). Fluorescence was determined using a Perkin-Elmer LS-5B luminescence spectrometer using an excitation wavelength of 355 nm and measuring emission at 440 nm, as described previously (31). Changes in fluorescence, as a function of BART addition, were then determined.

The theoretical best fit line described for the ∆F versus [BART] was obtained using a Michaelis-Menten, non-linear least squares fit of the data, as described in Nishimoto et al. (31). The Ki for a single binding site was determined using the following Equation 1, from Nomanbakh and Cerione (32).

$$
\frac{\Delta F}{\Delta F_{max}} = \frac{(K_i + L_F + R_T) - (K_i + L_F + R_T - L_F + R_T + 2R_T)}{L_F + R_T} \quad (Eq. 1)
$$

where ∆F is the fluorescence change after each addition of BART; ∆F_{max} is the maximal change in fluorescence at an extrapolated, infinite [BART]; K_i is the dissociation constant; L_F is the [BART], and R_T is the total concentration of ARL2/Mant-Gpp(NH)p.

**Immunoprecipitation**—Recombinant proteins or controls were incubated in single detergent buffer (SDB, 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) with appropriate nucleotides for 1 h at 4 °C with gentle rocking. Protein G-Sepharose (Amersham Pharmacia Biotech) beads (10 μl) were then added and incubated an additional 1 h. Beads were then washed 3 times with SDB. Specifically bound proteins were eluted by boiling beads in Laemmli’s sample buffer for 5 min. Samples were analyzed by immunoblotting, as described above, using rabbit polyclonal anti-BART (R46712-1) or mouse monoclonal anti-MYC epitope (33) as primary antibodies.

**Antibody Production**—Polyclonal rabbit antisera were raised against purified, recombinant BART (with the carboxyl-terminal 6His tag; R46712-1) or human ARL2 (R86336-1). Subcutaneous inoculations were made with a complete Freund’s adjuvant, followed by three boosts in incomplete Freund’s adjuvant. Preliminary characterization of the two antibody preparations has been primarily through immunoblotting. Less than 1 ng of purified BART-C-His and approximately 5 ng of ARL2 were detectable on immunoblots by the anti-BART and anti-ARL2 sera, respectively. The ARL2 antisera did not cross-react with other members of the ARF/ARL family. We tested 50 ng each of ARF1, ARF4, ARF6, ARL1, and ARL3. Immunoreactivity could be effectively and specifically competed by preincubation with recombinant antigen (commonly 15 μg of recombinant protein with 4 μl of serum).

**GAP Assay**—GTP hydrolysis of ARL2, in the presence and absence of BART, was analyzed as described previously (34). Briefly, ARL2 (2 μM final concentration) was loaded with [α-32P]GTP and then incubated in separate reactions +/− BART at 30 °C. Loading buffer consisted of 25 mM HEPES, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 0.1% Triton X-100, 1 mM dithiothreitol, 30 μCi of [α-32P]GTP, and 1 mM ATP. The GAP assay buffer included 1 mM GTP to lower the specific activity of free nucleotide and help prevent background levels to be raised by the presence of nucleotidases or other enzymes capable of hydrolyzing GTP in an ARL2-independent fashion. Reactions were stopped by dilution into ice-cold TNMD at various times and rapidly filtered on nitrocellulose filters (25 mm BA85, Schleicher & Schuell) to trap protein-bound nucleotides. Free nucleotides were then extracted from the filters by soaking in 1 M formic acid and resolved by chromatography on polyethyleneimine-cellulose thin layer plates developed in 1:1 (v/v) 1 M lithium chloride and 1 M formate. Controls were included to ensure that only relevant GTP was catalyzed by ARL2. Immunodetection was performed using a Molecular Dynamics Storm phosphorimaging system.

**Membrane Translocation Assay**—Translocation of proteins from cytosolic to membrane associated forms was monitored by a modification of the method of Donaldson et al. (35). The source of cytosol was the mouse BCS1H smooth muscle-like brain-tumor-derived cell line, ob-
The GTPase overlay assay detects a 19-kDa protein from bovine brain that binds ARL2-GTP but not ARL2-GDP. BART activity was partially purified from bovine brain by chromatography on DEAE-Sephaloc, as described under “Materials and Methods.” The pooled material (25 μg/lane) was resolved by denaturing gel electrophoresis, transferred to nitrocellulose, and assayed by the GTPase overlay assay, under various conditions, as indicated in the figure. Binding activity is only observed in the presence of the ARL2-[α-32P]GTP complex and is enhanced by the presence of mixed micelles of dimyristoylphosphatidylcholine and cholic acid/cholate. Separate filters were incubated with ARL2-[α-32P]GTP (lanes 1 and 2), dimyristoylphosphatidylcholine and cholic acid/cholate (lanes 2 and 4), [α-32P]GTP without ARL2 (lanes 3 and 4), or ARL2-[α-32P]GDP (lane 5).

RESULTS

Detection of an ARL2-binding Protein in Bovine Brain Lysates—The GTPase overlay assay was used to identify specific binding partners for activated ARL proteins. As described under “Materials and Methods,” this technique involves the electrophoretic separation of protein mixtures in SDS-polyacrylamide gels, followed by their transfer to nitrocellulose sheets where they are allowed to re-fold in a renaturation buffer and probed with a radioactively labeled ligand. This method depends upon the ability of the binding protein to (a) bind to the nitrocellulose, (b) be capable of re-folding properly following denaturing electrophoresis and transfer, (c) have a relatively high affinity for the ligand, and (d) be present in sufficient quantity to allow detection by the labeled ligand. Note that the ligand in this case is a protein, made radioactive by pre-loading with radionucleotides. Each of the human ARFs (ARF1–6) and two human ARLs (ARL2 and -3) were initially tested in the gel overlay assay, using bovine brain extract (an abundant source of ARF proteins) as tissue source for potential binding partners.

By using the gel overlay protocol, we detected specific association between ARL2-GTP and a protein from bovine brain that migrated on SDS gels with a predicted molecular mass of about 19 kDa (Fig. 1, lane 1). This binding activity was present in the soluble fraction (S100) but was absent from the pellet (P100; data not shown). Binding activity was increased approximately 20-fold or more by the inclusion of mixed micelles of dimyristoylphosphatidylcholine and cholic acid (D/C, Fig. 1, lane 2) in the binding buffer of the gel overlay assay. Triton X-100 (0.1%) could be used in place of D/C to similar effect (data not shown). The presence of D/C in the assay increased either re-folding of the binding protein or the interaction with ARL2 as it had only minimal effects on the binding of GTP to ARL2. We believe the former explanation is more likely true as Triton X-100 is not required in the solution binding assay (see below), but we have not explored this issue further.

Interaction between the binding protein and ARL2 was dependent on GTP being bound to ARL2, as no signal was observed when equal specific activity [α-32P]GDP replaced the [α-32P]GTP in the loading reaction (Fig. 1, lane 5). An important control is shown in Fig. 1, lanes 3 and 4. No binding activity was seen when the same amount of [α-32P]GTP, in the presence of ARL2, was used as the ligand in the overlay assay. This lack of binding activity was not changed by the addition of D/C (Fig. 1, lane 4). No GTP-binding proteins on the filter bound the labeled nucleotides in the overlay assay, due to the presence of high concentrations of unlabeled GDP and GTP in the binding buffer. The specific activity of the GTP in the ARL2-binding site is much higher than that of the nucleotide free in solution. Therefore, the GTPase overlay assay allowed the detection of a single protein species of approximately 19 kDa that bound ARL2 in a GTP-dependent fashion.

The specificity of the binding protein for different GTPases was determined by replacement of ARL2 with other ARFs (human ARF1, 3–6), ARL3, or the small GTPase RhoA. Each of these proteins was loaded with [α-32P]GTP, but none gave a signal in the overlay assay (data not shown) when fractions containing the ARL2 binding activity were tested. An estimate of the affinity between ARL2 and the binding protein was obtained from competition experiments (see Fig. 2). The specifically bound radioactivity in the gel overlay assay was effectively competed by the addition of increasing amounts of ARL2, loaded with unlabeled GTP. Half-maximal binding was observed with the addition of only 20 nM ARL2-GTP.

Whereas the binding activity was capable of re-folding after being boiled in Laemmli’s sample buffer, electrophoresis through polyacrylamide and out of the gel onto nitrocellulose, incubation of the nitrocellulose filter with trypsin (0.25 mg/ml for 2 h at RT) totally abolished the binding signal (data not shown).
shown). Taken together, these results indicate that the S100 fraction from bovine brain contains a protein that binds ARL2 in a GTP-dependent fashion and with high specificity, with an apparent $K_d$ in the low nanomolar range. This protein was named BART, for binder of Arl 2.

**Purification of BART from Bovine Brain—** Mouse tissues were used to check for the presence and relative abundance of BART activity in different organs. Tissues were harvested from a freshly sacrificed animal and homogenized in buffer, as described under “Materials and Methods.” Equal amounts of total tissue proteins (25 mg/lane) were loaded onto SDS-polyacrylamide gels and assayed with the GTPase overlay assay. The activity was most abundant in brain, with a lesser amount apparent in muscle tissue (data not shown). Thus, our earlier choice of bovine brain as source of the binding protein proved to be fortuitous. However, even in brain, BART appeared to be present in only low amounts. Assuming a 1:1 binding of BART and ARL2, we calculated BART to comprise only about 0.003% of soluble brain protein.

The overlay assay was used to follow BART activity during purification by column chromatography. As seen in Fig. 3, BART activity eluted as a single peak from a DEAE-Sepharose column, developed with an increasing gradient of sodium chloride. The position of the BART peak of activity corresponded to a concentration of 420 mM NaCl. Following concentration by ammonium sulfate precipitation, BART also eluted from the gel filtration column (AcA54 resin) as a single, monodisperse species with an apparent molecular mass (~25 kDa) consistent with the size determined from the gel overlay assay (data not shown). Thus, our earlier choice of bovine brain as source of the binding protein proved to be fortuitous. However, even in brain, BART appeared to be present in only low amounts. Assuming a 1:1 binding of BART and ARL2, we calculated BART to comprise only about 0.003% of soluble brain protein.

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Chromatography on the quaternary amine exchanger, Source Q, yielded another 7-fold enrichment with about 30% recovery. This was followed by high resolution reverse phase, C8 (Pro-RPC), chromatography that gave more than a 10-fold increase in specific activity with good recovery. At this point the BART preparation (shown in Table I) was enriched about 4000-fold from the S100 with an overall recovery of 4%. Comparison of activity in the gel overlay assay with the colloidal blue staining profile (see Fig. 4) revealed a single band in the 20-kDa region that exactly co-migrated with the ARL2-binding protein. We estimate that the BART was about 30% pure at this point. The band identified as BART was well separated from the 2–3 other bands staining in the gel (see Fig. 4, combined

### Table I

Representative purification of BART from bovine brain extracts

| Chromatographic step | Pooled protein | Purification | Recovery |
|----------------------|----------------|--------------|----------|
| S100                 | 6 g            | 1 ×          | 100      |
| DEAE-Sepharose       | 1.6 g          | 4 ×          | 75       |
| Ultrogel AcA54       | 337 mg         | 50 ×         | 30       |
| Source Q             | 970 μg         | 350 ×        | 10       |
| Pro-RPC              | 15 μg          | 4000 ×       | 4        |

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BART activity elutes from the Pro-RPC column with a clearly resolved protein band. BART activity eluting from the Source Q column (400 μg of protein) was pooled and applied to a 2-ml Pro-RPC column in 0.1% trifluoroacetic acid, as described under “Materials and Methods.” A, the column was developed with a rapid ramp to 30% acetonitrile, followed by a more gradual, linear gradient of 30–80% acetonitrile in 0.1% trifluoroacetic acid. A single peak of BART activity eluted at an acetonitrile concentration of approximately 60% acetonitrile. B, each fraction was resolved by SDS-polyacrylamide gel electrophoresis on a 12.5% acrylamide gel and stained with Coomassie Blue. C, proteins were transferred to nitrocellulose and assayed using the overlay binding assay. Total protein in the combined fractions 29 and 30 was determined to be 1.2 μg, of which 20–30% (~240–360 ng) was estimated to be the 19-kDa protein band. Note the similarities in relative staining intensity of the 19-kDa band in fractions 29/30 and 31 compared with the binding intensity in the same lanes.

Approximately 100 ng of the protein band at 19 kDa was excised from a 15% SDS-polyacrylamide gel and subjected to peptide sequence analysis. Partial trypptic cleavage allowed the purification of peptides by reverse phase high pressure liquid chromatography. Electrospray mass spectroscopy was then used to determine peptide sequences from two different peptides, 9 and 15. Phy. Homology searches of GenBank™ and Swiss-Prot protein databases failed to identify a single protein with high homology to human BART. However, each genomic sequence contains multiple stop codons within the BART ORF, as well as some other differences. The presence of a contiguous, genomic sequence highly homologous to BART-untranslated regions, without introns, is suggestive of processed pseudogenes. No good candidate for a functional BART gene was found.

In addition to the human ESTs, there are also a sufficient number of mouse ESTs available in the dbEST collection to allow the construction of a predicted, full-length mouse BART ORF. Alignment of the human and mouse BART sequences revealed them to be 96 and 85% identical at the protein and nucleotide levels, respectively (see Fig. 5A). The high degree of homology among such a large number of human ESTs, coming from diverse human cDNA libraries, is evidence for a single BART gene that is expressed in a diverse array of tissues and cells.

BART Is Ubiquitously Expressed in Human Tissues—Northern blot analysis of human tissues indicated that a single BART message is ubiquitous in all surveyed tissues. The ~2.0-kilobase pair transcript was most abundant in heart, brain, and skeletal muscle, with less message found in placenta, kidney, pancreas, lung, and liver (see Fig. 6). This result is very similar to those obtained from the screen for BART activity in mouse tissues where brain was the most abundant source for binding activity. With the development of rabbit polyclonal anti-BART sera, we were also able to screen for expression of BART in different mouse tissues, and we found a very similar pattern of expression to that suggested by Northern blotting but with brain the most abundant source (data not shown). The presence of ESTs encoding BART from cDNA libraries derived from even more human tissues is further evidence of the ubiquity of BART expression.

Characterization of Recombinant BART and Its Interaction with ARL2-GTP—PCR primers, directed against the 5’ and 3’ ends of the BART ORF, were used to amplify the full-length
coding region, using EST clone 505092 as template. The resulting 500-bp PCR product was subcloned into the bacterial expression vector pET3C for inducible expression of recombinant protein. The addition of a hexa-histidine tag at the carboxyl terminus led to greater stability of the recombinant protein in bacteria and facilitated purification. As shown in Fig. 7, IPTG induced the expression of a protein, readily detected by Coomassie Blue staining of bacterial lysates on SDS gels, that bound ARL2 \( \text{GTP} \) in a manner indistinguishable from bovine brain BART. This result confirmed that the peptides we had sequenced were from BART and that the consensus cDNA sequence from multiple ESTs (but amplified from one) encoded human BART.

In order to produce and readily purify sufficient amounts of recombinant human BART for analysis, a carboxyl-terminal hexa-histidine fusion protein (BART-6His) was expressed and purified via Ni\(^{2+}\) chelate chromatography. This method allowed approximately 25 mg of protein to be rapidly purified from 1 liter of bacterial culture. Unless otherwise stated, all subsequent work was performed with this fusion protein.

An inherent aspect of the GTPase overlay technique used to identify BART is protein denaturation, which is necessary to resolve electrophoretically individual components of the sample. To verify \textit{in vitro} interaction between BART and ARL2 in the absence of protein-denaturing agents, two approaches were taken. First, ARL2 was pre-loaded with the fluorescent GTP analog, Mant-Gpp(NH)p, and then titrated with BART or a buffer control containing BSA. As has been found for some other GTPase interactions (31, 32) complex formation between ARL2\( \text{GTP} \) and another protein caused variations in the microenvironment of the fluorophore, resulting in changes in emissions at 440 nm, after excitation at 355 nm. As shown in Fig. 8, additions of BART increased the Mant-Gpp(NH)p fluorescence over the control, indicative of association between ARL2\( \text{GTP} \) and BART. The change in fluorescence with varying concentrations of BART was used to determine a binding constant for the ARL2-BART interaction, as described under “Materials and Methods.” Values for the dissociation constant ranged between 10 and 20 nM in at least four different experiments, the same as that calculated by

[Fig. 5. Human BART cDNA sequence, including translated ORF and original tryptic peptides. A, consensus human BART cDNA sequence was determined by alignment of multiple overlapping EST clones and confirmed by direct DNA sequencing of two such clones (EST505092 and EST267702) which together contain the entire predicted BART cDNA. The 163-amino acid residues encoded by the BART coding region are indicated, and the two original tryptic peptides from bovine brain BART (with a single species difference in sequence given) are underlined. B, alignment of predicted human and mouse BART protein sequences. Overall identity between the two proteins is 95.7%.]

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competition in the GTPase overlay assay. This result confirmed that the high affinity interaction between ARL2
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GTP and BART in the GTPase overlay assay was not dependent on denaturation of BART or due to inappropriate refolding of BART on the nitrocellulose filter.

Another means of detecting specific interaction between ARL2 and BART was also employed. A GTPase-deficient mutant of ARL2, [Q70L]ARL2, was found to co-immunoprecipitate with BART in the presence of GDP but not in the presence of GTP. These results and a series of controls are shown in Fig. 9.

The demonstration of ARL2-BART binding in solution was also important for another reason. No protein has yet been shown to interact with any ARF protein in solution or, more specifically, in the absence of membranes/lipids. This is presumably because of the importance of membrane translocation of ARFs upon activation. Binding to membranes is thought to orient and promote protein interactions and has come to be thought of as a critical part of ARF actions. To begin to test for the role of membrane translocation in ARL2 or BART activation, we asked whether ARL2 or BART could be recruited to membranes upon activation of the ARL2 by the binding of GTP
\( g \), as described under “Materials and Methods.” BC3H1 cells were used as the source of cytosol as ARL2, BART, and ARFs (used as a positive control) were all present in readily detectable levels. After incubation in the presence of GDP for 20 min, the recruitment of ARF proteins was easily seen,
whereas no increase in the levels of ARL2 or BART was ever seen. This experiment was repeated at least three times with the same results (data not shown).

**BART Is Not an ARL2 GAP**—To investigate the effect of BART binding on ARL2, we first tested whether the protein was capable of increasing the intrinsic GTPase activity of ARL2, which is serving as an ARL2 GAP. Like ARF proteins, ARL2 has no detectable GTPase activity alone (14). Addition of BART did not alter the rate of GTP hydrolysis by ARL2 using the same conditions in which ARF GAP activity can be readily detected (data not shown; Ref. 34).

**DISCUSSION**

We describe the identification, purification, cloning, sequencing, and initial characterization of the first ARL-binding protein. BART binds specifically to ARL2 with an apparent $K_D$ of 20 nM and in a nucleotide (GTP)-dependent fashion. It is widely expressed in human and mouse tissues, with highest abundance in the brain. Although originally identified in a GTPase overlay assay, the specific interaction with ARL2 was also shown to occur in solution and without prior protein denaturation. Purified, recombinant BART was active as a high affinity, specific binder of ARL2-GTP but lacked any ARL2 GTPase-activating protein activity. Based on this spectrum of activities, we conclude that BART is an immediate downstream effector of ARL2. Further analysis of BART, its binding partners, and activities should assist in the elucidation of the physiological role of ARL2 and BART in cell regulation.

The sequence analysis of this first ARL-binding protein was disappointing in its lack of previously identified domains that may have provided insight into function or other binding partners. However, lack of conserved GTPase or other, more general, protein binding motifs is quite common among effectors for members of the ARF family. A cysteine-rich motif has been described in proteins with ARF GAP activity, which are also ARF effectors (5), but this ARF GAP domain is clearly absent on at least six other ARF-binding proteins, including PLD1, POR1/ARFaptin2, and the bacterial toxins CTA (cholera toxin) or LTA (Escherichia coli heat-labile toxin). So clues toward the identification of biological functions for ARL2 and BART must come from other sources.

The large number of ESTs encoding BART allowed the full-length sequence of the cDNA to be determined in silico and confirmed by DNA sequencing of selected clones. Genomic sequencing of human DNA revealed the presence of two BART-related genes, presumably pseudogenes. We conclude that there is a single BART message expressed in a wide array of cells and that the cDNA sequence shown in Fig. 5A is full length, or very nearly so.

It has become increasingly apparent that RAS-like GTPases often, if not always, interact with a large number (>10) of specific effectors. At least 13 different proteins bind to RAS in a GTP-sensitive manner (37), whereas RHO family members have at least 15 such binding partners (38). Although the underlying molecular mechanism(s) of most small GTPases remains uncertain, much of what is known regarding the pathways they regulate has come from the characterization of the proteins they bind. Based upon results with other GTPases, we fully expect there to be a number of ARL2-binding proteins, only one of which was identified by the GTPase overlay assay. Because this assay requires the GTPase binder to be denatured and renatured out of SDS while adhering to a nitrocellulose filter, it is likely that others do not survive these harsh constraints. The small size of BART was likely a contributing factor that facilitated its re-foiling on filters. The high affinity of active ARL2 for BART undoubtedly also was an important contributing factor in our ability to first detect, and later purify, the novel binding protein.

Although other ARL-binding proteins are predicted, having one in hand allows us to begin to test for function(s) and make comparisons to ARF proteins. A critical component in ARF signaling is thought to be the GTP-dependent membrane translocation that is evident for ARF1–5, although not ARF6 as it more stably associates with membranes. The covalent addition of myristate to the amino terminus of ARFs is a requirement for membrane binding and for ARF to function in cells (39). Thus, it was surprising when we first noted that recombinant ARL2, co-expressed in bacteria with N-myristoyltransferase, does not get acylated (21). This conclusion was further supported recently by the failure to detect any N-myristoylation of ARL2 in mammalian cells.5 Thus, it appears that ARL2 is distinct from ARF proteins in this regard, despite having the glycine at position 2 that is the site of acylation. Other structural differences between ARL2 and the ARFs may also explain why ARL2 binds guanine nucleotide triphosphates rapidly and to high stoichiometry. These characteristics are only found on an ARF protein after deletion of the amino-terminal o-helix. The inability of ARL2 or BART to bind to membranes after incubation with GTP-y-S distinguishes this GTPase from the ARF proteins and further supports the idea that ARL2 and BART bind in solution and may do so independently of membrane or lipids.

High affinity, GTP-specific interaction between BART and ARL2 has been demonstrated using three separate in vitro methods, and we have initiated studies designed to reveal aspects of the in vivo significance of this association. Preliminary studies indicate that BART and ARL2 can enter the nucleus,6 yet their sequences contain no identifiable nuclear localization sequence. Other studies appear to indicate a role for ARL2 in determination of Golgi morphology in at least some cell types,6 similar to what has been observed for ARF proteins, but a role for BART in this effect is uncertain. Additional work will be required before a testable model for ARL2 or BART action in cells can emerge. At about 50% identity in primary sequence, there is good reason to believe that the ARF proteins will provide a useful model to compare and contrast effects and models of ARLas in the future. These initial studies provide some evidence that interesting differences between the groups of GTPases in the ARF family will emerge. Since their discovery some 9 years ago, functional aspects of ARF proteins have been delayed by the extensive interest in the ARF proteins as regulators of membrane traffic and targets of the drug brefeldin A. It is anticipated that further characterization of BART, and identification and analysis of other ARF-specific effectors, will lead to a better understanding of the larger ARL branch of the ARF family of GTPases that may shed new light on the actions of ARF proteins as well.

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**REFERENCES**

1. Donaldson, J. G., and Klausner, R. D. (1994) *Curr. Opin. Cell Biol.* 6, 527–532
2. Nussler, C., and Balch, W. E. (1994) *Annu. Rev. Biochem.* 63, 949–990
3. Boman, A. L., and Kahn, R. A. (1995) *Trends Biochem. Sci.* 20, 147–150
4. Moss, J., and Vaughan, M. (1995) *J. Biol. Chem.* 270, 12327–12330
5. Zhang, C., Cavenagh, M. M., and Kahn, R. A. (1998) *J. Biol. Chem.* 273, 19792–19796

6 H. VanValkenburgh, J. D. Sharer, and R. A. Kahn, unpublished observations.

7 J. D. Sharer and R. A. Kahn, unpublished observations.

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4 A. L. Boman and R. A. Kahn, submitted for publication.
ARF-like 2-binding Protein, BART

6. Rudge, S. A., Cavenagh, M. M., Kamath, R., Sciorra, V. A., Morris, A. J., Kahn, R. A., and Engebret, J. (1998) Mol. Biol. Cell 9, 2025–2036
7. Stearns, T., Kahn, R. A., Botstein, D., and Hoyt, M. A. (1990) Mol. Cell. Biol. 10, 6690–6699
8. Stearns, T., Willingham, M. C., Botstein, D., and Kahn, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1238–1242
9. Franco, M., Loretto, J., Robin, S., Monier, S., Goud, B., Chardin, P., and Chavrier, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9926–9931
10. Goldberg, J. (1998) Mol. Cell. Biol. 18, 7038–7051
11. Cukierman, E., Huber, I., Rotman, M., and Cassel, D. (1995) Science 270, 1999–2002
12. Brown, M. T., Andrade, J., Radhakrishna, H., Donaldson, J. G., Cooper, J. A., and Randazzo, P. A. (1998) Mol. Cell. Biol. 18, 7038–7051
13. Poon, P. P., Cassel, D., Spang, A., Rotman, M., Pick, E., Singer, R. A., and Johnston, G. C. (1999) EMBO J. 18, 555–564
14. Clark, J., Moore, L., Krasinskas, A., Way, J., Battey, J., Tamkun, J., and Kahn, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8952–8956
15. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
16. Kahn, R. A., Kern, F. G., Clark, J., Gelmann, E. P., and Rulka, C. (1991) J. Biol. Chem. 266, 2606–2614
17. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Science 263, 523–529
18. Hong, J. X., Lee, P. J., Patton, W. A., Lin, C. Y., Moss, J., and Vaughan, M. (1998) J. Biol. Chem. 275, 15872–15876
19. Tamkun, J. W., Kahn, R. A., Kissinger, M., Brizuela, B. J., Rulka, C., Scott, M. P., and Kennison, J. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3120–3124
20. Lee, F. J., Huang, C. F., Yu, W. L., Bux, L. M., Lin, C. Y., Huang, M. C., Moss, J., and Vaughan, M. (1997) J. Biol. Chem. 272, 30998–31005
21. Randazzo, P. A., Weiss, O., and Kahn, R. A. (1995) Methods Enzymol. 257, 128–135
22. Clark, J., Moore, L., Krasinskas, A., Way, J., Battey, J., Tamkun, J., and Kahn, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8952–8956
23. Manser, E., Leung, T., Monfries, C., Teo, M., Hall, C., and Lim, L. (1992) J. Biol. Chem. 267, 16025–16028
24. Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994) Nature 376, 40–46
25. Lounsbury, K. M., Beddow, A. L., and Macara, I. G. (1994) J. Biol. Chem. 269, 11285–11290
26. Laemmli, U. K. (1970) Nature 227, 680–685
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
28. Zhang, C. J., Rosenwald, A. G., Willingham, M. C., Skunz, S., Clark, J., and Kahn, R. A. (1994) J. Cell Biol. 124, 289–300
29. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
31. Nisimoto, Y., Freeman, J. L. R., Motalebi, S. A., Hirshberg, M., and Lambeth, J. D. (1997) J. Biol. Chem. 272, 18834–18841
32. Nomanbhoy, T. K., and Cerione, R. (1996) J. Biol. Chem. 271, 10004–10009
33. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
34. Randazzo, P. A., and Kahn, R. A. (1994) J. Biol. Chem. 269, 10758–10763
35. Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6408–6412
36. Balch, W. E., Dunphy, W. G., Braell, W. A., and Rothman, J. E. (1984) Cell 39, 405–416
37. Campbell, S. L., Khoosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998) Oncogene 17, 1395–1413
38. Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102
39. Kahn, R. A., Clark, J., Boman, A. L., Stearns, T., Zhang, C., Randazzo, P. A., Terui, T., and Cavenagh, M. (1995) J. Biol. Chem. 270, 143–150
40. Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) J. Biol. Chem. 271, 21767–21774