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ARL2 and BART Enter Mitochondria and Bind the Adenine Nucleotide Transporter

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The ADP-ribosylation factor-like 2 (ARL2) GTPase and its binding partner binder of ARL2 (BART) are ubiquitously expressed in rodent and human tissues and are most abundant in brain. Both ARL2 and BART are predominantly cytosolic, but a pool of each was found associated with mitochondria in a protease-resistant form. ARL2 was found to lack covalent N-myristoylation, present on all other members of the ARF family, thereby preserving the N-terminal amphipathic α-helix as a potential mitochondrial import sequence. An overlay assay was developed to identify binding partners for the BART·ARL2·GTP complex and revealed a specific interaction with a protein in bovine brain mitochondria. Purification and partial microsequencing identified the protein as an adenine nucleotide transporter (ANT). The overlay assay was performed on mitochondria isolated from five different tissues from either wild-type or transgenic mice deleted for ANT1. Results confirmed that ANT1 is the predominant binding partner for the BART·ARL2·GTP complex and that the structurally homologous ANT2 protein does not bind the complex. Cardiac and skeletal muscle mitochondria from *ant1*$^{-}$*/ant1*$^{-}$ mice had increased levels of ARL2, relative to that seen in mitochondria from wild-type animals. We conclude that the amount of ARL2 in mitochondria is subject to regulation via an ANT1-sensitive pathway in muscle tissues.

INTRODUCTION

ADP-ribosylation factor-like 2 (ARL2) is a 21-kDa GTPase that shares 45% primary sequence identity with ADP-ribosylation factor 1 (ARF1; Clark *et al.*, 1993). Like ARFs, the ARL2 message is expressed in all mammalian tissues (Clark *et al.*, 1993) and achieves its highest levels in neural tissue. The ARL2 protein has close structural homologs in other metazoans such as *Drosophila melanogaster* (80% identity) and *Caenorhabditis elegans* (60% identity). Despite their structural similarities, ARLs lack the biochemical and genetic activities that define the ARF proteins, including cofactor activity for cholera toxin, stimulation of phospholipase D, and complementation of an *arf1*− *arf2*− double deletion in *Saccharomyces cerevisiae* (Kahn *et al.*, 1991; Brown *et al.*, 1993; Cockcroft *et al.*, 1994). ARL2 is notable in the ARF family in that it binds GDP or GTP rapidly and to high stoichiometry, even in the absence of added lipids or detergents (Clark *et al.*, 1993). This property probably contributed to the successful use of a gel overlay assay to identify and purify binder of ARL2 (BART), a specific binder of ARL2 (Sharer and Kahn, 1999). In contrast to the roles for ARFs in vesicular traffic (Stearns *et al.*, 1990; Rothman, 1994; Boman and Kahn, 1995), ARL2 has been implicated as a regulator of tubulin dynamics in mammalian cells (Bhamidipati *et al.*, 2000), cytokinesis in plant cells (McElver *et al.*, 2000), and vulval development in *C. elegans* (Antoshechkin and Han, personal communication).

BART is a soluble 19-kDa protein originally identified and purified from bovine brain. The binding of ARL2 to BART is of high affinity ($\leq$20 nM) and dependent on the binding of GTP to ARL2 (Sharer and Kahn, 1999). Because BART lacks ARL2 GTPase-activating protein activity and its tissue expression extensively overlaps with that of ARL2 (Sharer and Kahn, unpublished data) it is likely that BART is the first effector identified for ARL2.

Primary sites of action for regulatory GTPases in the RAS superfamily include the plasma membrane (e.g., RAS and RHO), the membranes of the secretory pathway (e.g., Golgi complex; ARFs, and RABs), and the nucleus (RAN). However, no GTPases in the RAS superfamily have previously been identified in mitochondria. In the studies described below, we provide evidence that a portion of ARL2 is in mitochondria and that it binds through its effector, BART, to the adenine nucleotide transporter (ANT).

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ANT proteins are abundant, integral components of the mitochondrial inner membrane, where they homodimerize and form channels used to exchange ATP and ADP, thereby controlling the levels of ATP in the cytoplasm (Fiore *et al.*, 1998). Mammals possess multiple ANT isoforms, with two found in rodents and three in cows and humans (Powell *et al.*, 1989; Chen *et al.*, 1990; Shinohara *et al.*, 1993; Fiore *et al.*, 1998). The different isoforms share ~88% primary sequence identity but exhibit distinct tissue-specific expression patterns (Stepien *et al.*, 1992; Graham *et al.*, 1997; Levy *et al.*, 2000). In rodents, ANT1 is the major isoform present in skeletal and cardiac muscle, and it is also abundant in brain (Stepien *et al.*, 1992; Graham *et al.*, 1997; Levy *et al.*, 2000). In contrast, murine ANT2 is more widespread than ANT1 but is less prevalent in heart and skeletal muscle (Stepien *et al.*, 1992; Graham *et al.*, 1997; Levy *et al.*, 2000). ANT1, but not ANT2, has also been the subject of considerable interest for its apparent role as a component of the permeability transition pore, the formation of which appears to be a critical early step in some forms of apoptosis (Crompton, 2000a,b; Kroemer and Reed, 2000). Regulation of ANT1 functions, particularly adenine nucleotide transport, has not been described, in large part perhaps because of its inaccessibility in the intact organelle.

Previous reports have described the presence of multiple species of GTP-binding proteins in mitochondria from rat liver (Lithgow *et al.*, 1991; Cortese, 1999) or bovine adrenal cortex (Thomson *et al.*, 1995; Sleer and Hall, 2000). Two of these proteins localized to the outer mitochondrial membrane, whereas the others were found at inner/outer membrane contact points. Roles for GTPases in regulation of mitochondrial membrane fusion (Cortese, 1999), preprotein import, steroid hormone production, and aggregation of mitochondria during spermatogenesis (Thomson, 1998) have been suggested, although experimental evidence for some of these functions is lacking.

MATERIALS AND METHODS

Antibodies

Polyclonal antisera were raised in rabbits against recombinant ARL2 (R-86336) or a BART-His_6 fusion protein (R-46712) as previously described (Sharer and Kahn, 1999). Each of these antibodies is sufficiently sensitive to detect ≤1 ng of purified, recombinant protein by immunoblotting. The specificity of the ARL2 antibody was tested against human ARF1–6, ARL1, and ARL3 with no cross-reactivity evident in immunoblots. Each was affinity purified in two steps. Immunoglobulins were first enriched on a 1-ml HiTrap Protein G column (Amersham Biosciences, Piscataway, NJ). The specific antibodies were then affinity purified by passing over antigen-specific resins, composed of ARL2 or BART-His_6 covalently bound to Affigel 15 beads (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Polyclonal antibodies specific for rodent ANT1 and ANT2 have been described previously (Graham *et al.*, 1997). Monoclonal antibodies specific to matrix heat-shock protein 70 (mtHSP70) or cytochrome *c* were obtained from Affinity Bioreagents (Golden, CO) and Trevigen (Gaithersburg, MD), respectively.

Gel Electrophoresis and Immunoblotting

Protein samples (20–40 μg of total protein/lane) were boiled in Laemmli's sample buffer for 5 min before electrophoresis on 12.5 or 15% discontinuous polyacrylamide gels (Laemmli, 1970). Resolved proteins were electrophoretically transferred to nitrocellulose and immunoblotted as described (Towbin *et al.*, 1979; Zhang *et al.*, 1994) by using affinity-purified R-86336 or R-46712 at 180 or 150 ng/ml, respectively. Immunoreactive proteins were visualized using enhanced chemiluminescence detection (PerkinElmer Life Sciences, Boston, MA).

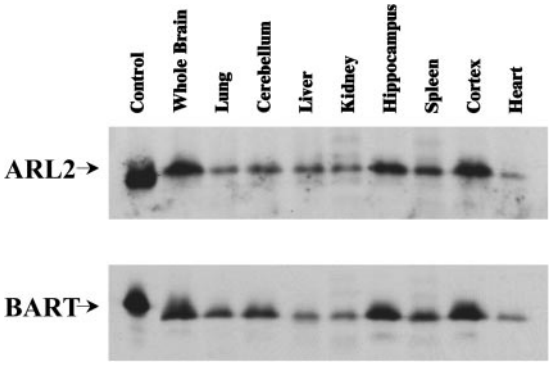

Figure 1. ARL2 and BART are expressed to the highest levels in neural tissues. The indicated rat tissue homogenates (25 μg of total protein/lane) were assayed by immunoblot for the presence of ARL2 and BART, as described under MATERIALS AND METHODS. Recombinant ARL2 (5 ng) or BART-His_6 (2 ng) was included as controls on the appropriate gel. This experiment was repeated multiple times with rat and mouse tissues and always gave similar results. Slight differences in mobility between controls and tissue lysates are discussed under MATERIALS AND METHODS.

Note that the recombinant BART used as a positive control in immunoblots contains a poly-histidine tag that makes it migrate slightly slower in SDS gels than that from cells or tissues (Figure 1). It was also noted that ARL2, either recombinant or in cell or tissue lysates, can migrate as a doublet (Figures 1 or 3). No covalent modifications or proteolytic processing is known to occur on ARL2, so no explanation for the doublet is currently available. However, there was no consistent difference in behavior of the two ARL2 immunoreactive bands with regard to any of the results described herein.

Cell Culture

Sf295 glioblastoma cells (obtained from the National Cancer Institute tumor cell line repository) and normal rat kidney (NRK) cells were grown in RPMI containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in 10% CO_2. For immunocytochemistry, cells were seeded at a density of ~1×10^5 cells/ml on glass coverslips and grown to 50–80% confluence before collection and processing. For subcellular fractionation studies, ~5×10^5 cells were seeded into ten 150-mm^2 dishes and allowed to reach 80% confluence. Cells were then harvested by scraping with a Teflon cell scraper in phosphate-buffered saline (PBS) and pelleted before further treatment. For transient transfection experiments, NRK cells were seeded at a density of 1×10^5 cells/ml on glass coverslips and incubated for 24 h before transfection with pcARL2myc (pcDNA3 containing the ARL2 coding region fused to a C-terminal myc epitope) by using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN), as per manufacturer's instructions.

Fluorescence Microscopy

Cells were prepared for immunocytochemistry as previously described (Zhang *et al.*, 1994). All cells were viewed and images captured with an Olympus BX60 epifluorescence microscope fitted with a Dage-MTI model charge-coupled device-300-RC camera and Image Pro capturing and imaging software. Labeling of mitochon-

dria with Mitotracker (Molecular Probes, Eugene, OR) was achieved by incubating cells in culture with 0.2 μM Mitotracker for 30 min before fixation.

Subcellular Fractionation/Isolation of Mitochondria

All subcellular fractionation and organelle isolation techniques were adapted from previously reported, well established methods (Greenawalt, 1974; Graham *et al.*, 1994; Spector *et al.*, 1998). All steps were carried out at 4°C. Sf295 cells were collected by centrifugation and washed once with PBS and twice with mitochondrial isolation buffer (10 mM HEPES pH 7.4, 70 mM sucrose, 200 mM mannitol, 1 mM EDTA, protease inhibitor cocktail [Sigma, St. Louis, MO]; Greenawalt, 1974). Cells were then resuspended in mitochondria isolation buffer, passed five times through a 23-gauge needle, and disrupted by N_2 cavitation (25 min at 500 psi). The cell lysate was then subjected to centrifugation at 1000 × *g* for 10 min, yielding a nuclear pellet and postnuclear supernatant. The pellet was carefully resuspended in isolation buffer, by using either a large-bore pipette or a few strokes with a loose-fitting Dounce homogenizer, and collected again by centrifugation at 1000 × *g*, yielding the final nuclear pellet. The heavy mitochondrial fraction was obtained from the postnuclear supernatant after centrifugation at 3000 × *g* for 10 min. This pellet was resuspended and the 3000 × *g* spin was repeated to obtain the final heavy mitochondrial pellet. The supernatant from the 3000 × *g* spins was then subjected to 15,000 × *g* for 10 min. The resulting light mitochondrial pellet was resuspended and sequential 3000 and 15,000 × *g* spins yielded the final light mitochondrial pellet. Finally, the 15,000 × *g* supernatant was further fractionated at 100,000 × *g* for 60 min, yielding the 100,000 × *g* supernatant (S100) and pellet (P100).

Three different methods were used to further enrich for mitochondria after preparation of the crude mitochondrial fraction by differential centrifugation. For additional purification of mitochondria, velocity or equilibrium density gradient centrifugation was used. For rapid isolation of purified mitochondria, heavy or combined heavy and light mitochondria were applied to 30% Percoll in mitochondria isolation buffer and resolved at 95,000 × *g* for 30 min. The lower portion of the dense mitochondrial band was removed with a Pasteur pipette and washed twice with mitochondria isolation buffer (Hovius *et al.*, 1990). Alternatively, heavy or combined heavy and light mitochondria were adjusted to 35% Optiprep (Iodixanol, Nycodenz) and applied below a continuous 10–30% Optiprep gradient and spun at 52,000 × *g* for 90 min (Graham *et al.*, 1994). The gradients were fractionated from the bottom by using a Bio-Rad peristaltic pump attached to a capillary tube, and equal-volume samples were assayed in immunoblots. In the third scheme, mitochondria were fractionated on a discontinuous sucrose gradient (Spector *et al.*, 1998), and fractions were collected for analysis as described above. Markers of other organelles (e.g., AP-2 for plasma membranes) were used to confirm that fractionation was occurring as previously described, so complete descriptions of the fractionation of the other organelles were not performed.

For fractionation of mammalian tissues, adult cow brains (Pel-Freeze) or freshly excised organs from fasted mice or rats were washed in ice cold mitochondria isolation buffer, minced with surgical scissors, and homogenized with a chilled Teflon-glass homogenizer. The crude lysate was filtered through five layers of cheesecloth and processed for subcellular fractions as described above for cultured cells.

Test for N-myristoylation

Target proteins were coexpressed in *Escherichia coli* with N-myristoyltransferase (NMT) as described previously (Duronio *et al.*, 1990; Randazzo and Kahn, 1995; Van Valkenburgh *et al.*, 2001). Briefly, cultures were grown to an A_{600} of ~0.6 in Luria broth, at which time 1 mM isopropyl β-D-thiogalactoside and 40 μCi/ml [^{3}H]myristic acid were added and cultures were allowed to grow for an additional 90 min. Cells were collected by centrifugation and resuspended in SDS sample buffer. Proteins were resolved on a 15% SDS-polyacrylamide gel (50 μg of protein/lane). The gel was then treated for fluorography by using Enhance (Amersham Biosciences), dried, and exposed to film. Plasmids used for protein expression include the following: pBB131, yeast NMT (Duronio *et al.*, 1990); pNMT1 (Randazzo and Kahn, 1995), N-terminal truncation of human NMT1; pHV641 (Van Valkenburgh and Kahn, 2001), full-length human NMT1; pHV644 (Van Valkenburgh and Kahn, 2001), full-length human NMT2; pJCY1–74 (Kahn *et al.*, 1995), yeast ARF1; pJCY1–75 (Kahn *et al.*, 1995), yeast [G2A]ARF1; pOW12 (Weiss *et al.*, 1989), human ARF1; and pJCH14-4 (Clark *et al.*, 1993), human ARL2.

For expression and analysis in mammalian cells (Jones *et al.*, 1990), Chinese hamster ovary (CHO) cells were transiently transfected using FuGENE6 with either pcDNA3 (empty vector), pHV728 (hARF5-myc/pcDNA3), or pHV305 (hARL2-myc/pcDNA3) in the presence of 200 μCi/ml [^{3}H]myristic acid. Each of these expression constructs contains the full-length protein fused at the C terminus to the 10-residue myc epitope and cloned into the *Bam*HI and *Xba*I sites of pcDNA3 (Invitrogen). Sixteen hours after transfection, cells were washed with PBS, collected by scraping in lysis buffer (0.01 M Na phosphate pH 7.4, 0.9% NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 0.2% Na azide, 95 mM NaF). Cells were lysed by passage through a 21-gauge needle and antibody 9E10 (anti-myc) was added. After overnight incubation, protein G Sepharose (Amersham Biosciences) was added and incubated for 1 h. Beads were collected by centrifugation and washed two times in lysis buffer. SDS sample buffer was added to each sample and boiled. Samples were resolved by SDS-PAGE, and fluorography was performed as described above.

Treatments of Mitochondria

Protease sensitivity was determined using freshly prepared mitochondria and S100 from rat brain. Thermolysin (Sigma) was added to 40–60 μg of brain proteins at a ratio of 1:5 and incubated at 30°C for 10 min. Reactions were terminated by the addition of protease inhibitors and controls included the addition of the inhibitors before protease.

Fractionation of mitochondria was achieved by sequential treatment of freshly isolated rat brain mitochondria with low (0.1) and high (0.25) digitonin (Calbiochem, San Diego, CA):protein ratios for 15 min at 0°C. The higher concentration of digitonin has been previously shown (Hovius *et al.*, 1990) to selectively extract proteins from the outer mitochondrial membrane and leave the inner membrane largely intact.

Overlay Assays

Binding of the ARL2(GTP)·BART complex or in vitro translated ARL2 and/or BART to other proteins was assayed using a modified version of the gel overlay assay described in Sharer and Kahn (1999). For assays using radioactive GTP, recombinant ARL2 (12 μM) was loaded with 8 μCi of [α-^{32}P]GTP in 10 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.1, 1 mM EDTA, 1.5 mg/ml bovine serum albumin, 0.5 mM magnesium acetate, 0.1% Triton X-100 for 10 min at 30°C. The buffer was then increased to 2 mM magnesium acetate, and BART-His_6 (19 μM) was added and incubated for 10 min. Potential binding proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose. Proteins were renatured by incubating the filter in renaturation buffer [10 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.1, 100 mM potassium acetate, 5 mM magnesium acetate, 0.25% Tween 20, 5 mM dithiothreitol, 0.1% Triton X-100, and 0.5% bovine serum albumin] for at least 1 h before the ARL-[α-^{32}P]GTP-BART mixture was added. After 15 min at room temperature, the filter was washed three times with ~30 ml of binding buffer before exposure to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).

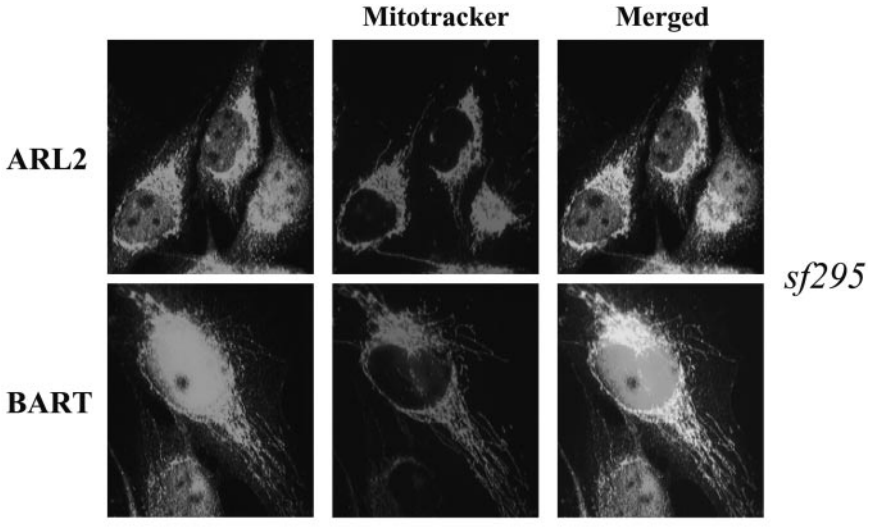

Figure 2. ARL2 and BART localize to mitochondria by indirect immunofluorescence. Subconfluent sf295 glioblastoma cells were processed for indirect immunofluorescence microscopy, by using ARL2 or BART polyclonal antibodies (left panels) and Mitotracker (middle panels), as described under MATERIALS AND METHODS. The merged images on the right reveal the extensive overlap in staining of ARL2 and BART with the vital mitochondrial stain.

For analysis with in vitro translated ARL2 or BART, the coding regions in pcDNA3 (5 μg of DNA/112-μl reaction; Sharer and Kahn, 1999) were transcribed and translated (TNT quick-coupled translation kit; Promega, Madison, WI) in the presence of [^{35}S]methionine and [^{35}S]cysteine (ICN Biomedicals, Cleveland, OH). Expression of radiolabeled ARL2 or BART was confirmed by autoradiography.

Protein Purification and Microsequencing

Bovine brain mitochondrial proteins (~340 μg) were incubated in 0.1 M Na_2CO_3 pH 11.5. After 60 min at 4°C, the mitochondria were collected and boiled in SDS sample buffer, and proteins were resolved by SDS-PAGE. Two identical gels were used to determine ARL2·BART binding in the gel overlay assay and to identify the corresponding band after staining with colloidal brilliant blue (Sigma). The latter gel was extensively washed in 25% methanol to remove SDS, and ~40 pmol of the 32-kDa protein was then excised and submitted to the Yale Cancer Center Mass Spectrometry Resource and W.M. Keck Foundation Resource Laboratory for analysis. Tryptic peptides were subjected to Q-TOF MS/MS analysis.

RESULTS

ARL2 and BART Are Most Abundant in Brain and Derived Cell Lines

Human and rat ARL2 share 95% amino acid sequence identity, and human BART and a translated mouse expressed sequence tag clone encoding a predicted BART protein are also 95% identical. Polyclonal antibodies raised against recombinant human ARL2 or BART were used to determine the levels of protein expression. Each antiserum binds rat or human ARL2 or BART with essentially the same sensitivity and specificity.

Expression patterns for ARL2 and BART were very similar in a number of rat (Figure 1) and mouse tissues and in several human, mouse, or rat cell lines. Thus, the levels of expression of the two proteins in different tissues have also been conserved in mammals. Although ARL2 and BART were detected in all tissues examined, each was particularly abundant in brain, especially the hippocampus and cortex, with somewhat less in the cerebellum. We estimate that each protein was present in these tissues at ~0.01% of total cell protein. Considerably less total protein was detected in heart. The latter result differs somewhat from Northern blot analyses, in which levels of BART mRNA in heart were nearly as high as in brain (Sharer and Kahn, 1999). Screening of a variety of cultured mammalian cell lines for endogenous ARL2 and BART revealed relatively high levels of each protein in tumor cell lines of neuronal origin (e.g., sf295 glioblastoma or SK-N-SH neuroblastoma), consistent with the results of the tissue screens. In contrast, several commonly used cell lines (e.g., CHO, NRK, and monkey kidney [COS-7] cells) contained little, if any, detectable protein ($\leq$1 ng/25 μg of total cell protein). Overall, the distribution pattern of the two proteins in mammalian tissues and cell lines, and their relative abundance, were remarkably similar. Sf295 cells grow as adherent, flattened cells that facilitate immunocytochemistry, so they were chosen for more detailed analyses.

ARL2 and BART Localize to Mitochondria

The subcellular localization of endogenous ARL2 and BART was initially investigated in sf295 cells by using indirect immunofluorescence. ARL2 or BART antisera produced distinctive mitochondrial labeling (Figure 2), as indicated by colocalization with the vital mitochondrial stain Mitotracker, or with antibodies against cytochrome *c* or matrix heat-shock protein 70 (our unpublished data). Staining of mitochondria by each of the four antisera was evident in sf295 cells permeabilized with Triton X-100 (0.1%) but not in those permeabilized with saponin (0.2%). This is consistent with the conclusion that each antigen is inside mitochondria, because mitochondrial membranes are known to be low in cholesterol and are thus poorly permeabilized by saponin. In contrast to sf295 cells, little or no specific staining of endogenous ARL2 or BART was evident in NRK, CHO, or COS-7 cells, consistent with the relatively low levels of the two proteins in these cells, as detected by immunoblot analyses.

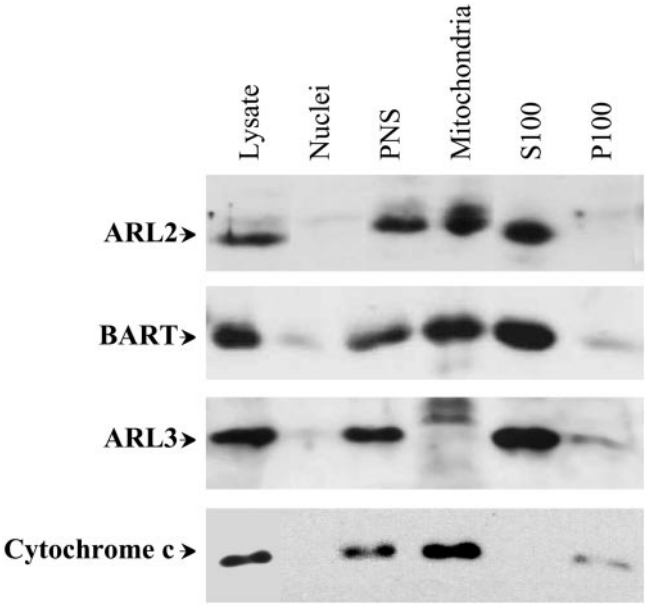

Figure 3. Subpopulation of endogenous ARL2 and BART cofractionates with mitochondria from sf295 cells. Subcellular fractionation of sf295 cells was performed as described under MATERIALS AND METHODS. Equal protein samples (25 μg) from each fraction were then analyzed by Western blotting with ARL2, BART, ARL3, and cytochrome *c* antibodies. ARL2, BART, and cytochrome *c* are present in the mitochondria-enriched fraction, whereas ARL3 is absent from this material. Also, note that there was much more total protein in the S100 than mitochondria fractions, such that we estimate that 10–20% of cellular ARL2 and BART are associated with mitochondria in sf295 cells. Similar results were obtained at least twice and when using rat brain tissue in separate isolation procedures.

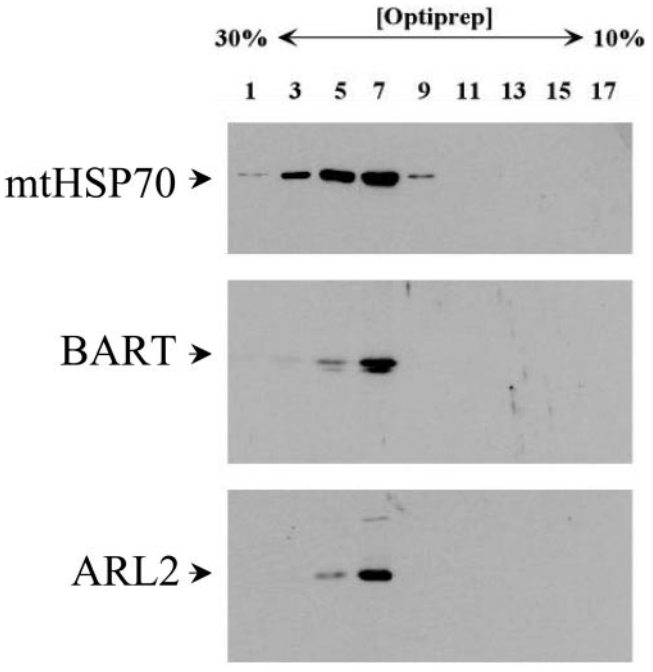

Figure 4. ARL2 and BART cosediment with the inner matrix marker mtHSP70 after density gradient centrifugation. Heavy mitochondria were isolated from sf295 cells by differential centrifugation, applied beneath a continuous 10–30% Optiprep (Iodixanol) gradient, and centrifuged at 52,000 × *g* for 90 min, as described under MATERIALS AND METHODS. The gradient was then fractionated (bottom to top) and equal-volume samples from individual fractions were analyzed by immunoblot with the indicated antibodies. Similar results were obtained multiple times and by using combined light and heavy mitochondria from sf295 cells or rat brains.

Subcellular Fractionation Confirms the Association of ARL2 and BART with Mitochondria

To confirm and extend the location of ARL2 and BART in cultured cells, sf295 cells were analyzed by subcellular fractionation. Cells were harvested, lysed, and subjected to differential centrifugation to enrich for specific cellular components. The resulting fractions were then assayed by immunoblot analyses for the presence of ARL2, BART, ARL3 (53% identical to ARL2), or cytochrome *c* (a marker for mitochondria). All four proteins were readily detected in total cell lysates (25 μg of protein/lane) and were excluded from the nuclear fraction (Figure 3). ARL2, BART, and cytochrome *c* were each enriched in the mitochondria fraction, but ARL3 was not. In contrast, 80–90% of ARL2, ARL3, and BART, but not cytochrome *c*, were present in the 100,000 × *g* supernatant (S100). Little or none of the four proteins appeared in the high-speed pellet, which consisted primarily of membrane vesicles.

The mitochondria examined herein consisted of combined heavy (3000 × *g* pellet) and light (15,000 × *g* pellet) mitochondria fractions. When examined separately, ARL2 and BART were each detected in both pellets (our unpublished data). The heavy mitochondria fraction is generally of higher purity, whereas the light mitochondria fraction is known to be contaminated with components of the Golgi apparatus, endoplasmic reticulum, lysosomes, and peroxisomes. One or both of these fractions are routinely used as a starting point in the isolation of highly enriched mitochondria preparations.

The heavy mitochondria fraction from sf295 cells was applied to linear 10–30% gradients of Optiprep (Graham *et al.*, 1994). After centrifugation, fractions were collected and analyzed by immunoblotting for ARL2, BART, or mtHSP70 (Figure 4). The ARL2 and BART continued to cofractionate with the mitochondrial matrix marker mtHSP70. Previously described contaminants of the combined heavy and light mitochondria pooled fractions (e.g., plasma membranes identified by blotting with AP-2 antisera) were seen to be clearly resolved from mtHSP70, ARL2, and BART (our unpublished data). Because soluble proteins such as ARL2 and BART were unable to enter the gradient unless they were associated with a larger structure, these data indicate that fractions of the ARL2 and BART were stably associated with mitochondria from sf295 cells. Identical results were obtained when rat brain tissue was fractionated in the same way. Fractionation on linear sucrose density gradients and in 30% Percoll (see MATERIALS AND METHODS) were performed separately and also revealed complete cofractionation of a similar sized fraction of the total cellular ARL2 and BART with mitochondrial markers.

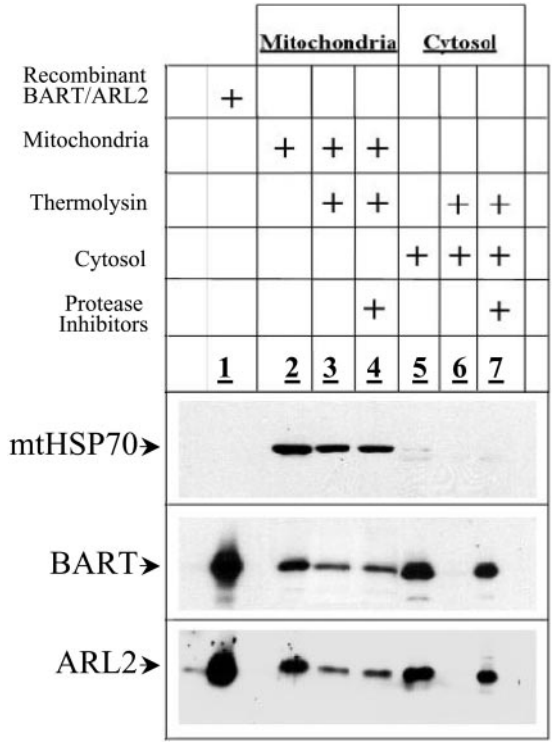

Figure 5. Mitochondrial ARL2 and BART are resistant to protease digestion. Mitochondria (lanes 2–4) and cytosol (S100; lanes 5–7) were isolated from sf295 cells by differential centrifugation and those in lanes 2, 3, 6, and 7 were treated with thermolysin +/− protease inhibitors, as described in detail under MATERIALS AND METHODS. Mitochondria were then collected by centrifugation and proteins extracted by boiling in SDS sample buffer. Proteins (25 μg) were resolved by SDS-PAGE and probed with the indicated antibodies by immunoblot analyses. Some lysis or loss of mitochondria appeared to have occurred during incubation and reisolation, because some loss of each protein is evident (compare lane 2 to lane 3 or 4). Purified recombinant ARL2 (5 ng) or BART (2 ng) was included as positive controls on separate gels, shown in lane 1. These results are representative of at least three separate experiments.

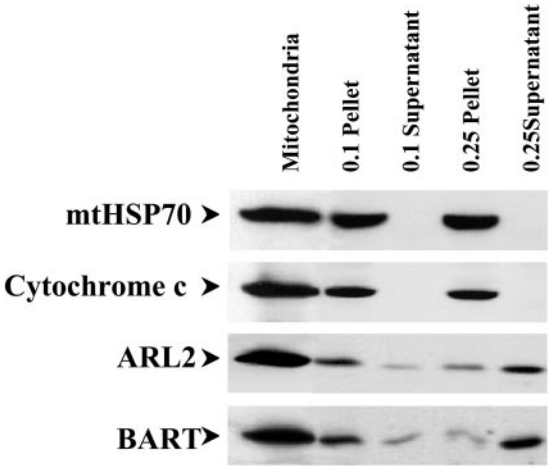

Figure 6. ARL2 and BART behave as mitochondrial intermembrane proteins. Purified rat brain mitochondria (lane 1) were treated with digitonin at a protein:detergent ratio of 0.1 or 0.25 before separating soluble and particulate fractions, as described under MATERIALS AND METHODS. Immunoblots were performed as described previously, by using antibodies specific for mtHSP70 (a marker for matrix protein retention), cytochrome *c* (a marker for the inner membrane), ARL2, or BART. These results are representative of at least three separate experiments.

ARL2 and BART Localize within Mitochondria

Given the extensive literature on the binding of ARFs to Golgi and other membranes, and their role in coat protein recruitment, we anticipated finding the ARL2 and BART on the cytoplasmic surface of the outer mitochondrial membrane. However, treatment of purified, intact sf295 mitochondria with high salt (0.5 M NaCl) or alkaline carbonate (0.1 M Na_2CO_3, pH 11.5), which efficiently removes peripherally bound proteins from biological membranes (Cavenagh *et al.*, 1996), failed to extract the ARL2 or BART (our unpublished data). Protease sensitivities of the ARL2, BART, and mtHSP70 associated with purified mitochondria were also examined and compared with that of ARL2 and BART in the S100 fraction (Figure 5). Treatment of the S100 fraction with thermolysin (5:1 for 10 min at 30°C) resulted in the complete degradation of the proteins, whereas the ARL2, BART, and mtHSP70 in the mitochondrial fraction were all resistant to the protease (Figure 5). Partial or complete solubilization of the organelles with detergent rendered the otherwise resistant ARL2 and BART susceptible to protease digestion (our unpublished data). The same results were obtained when purified rat brain mitochondria were used as the source. These data are consistent with the conclusion that this fraction of ARL2 and BART is not extrinsic, cytosolic-facing proteins but resides inside mitochondria.

To identify where ARL2 and BART localize within mitochondria we performed differential detergent extractions studies. Progressive disruption of the outer mitochondrial membrane has been observed when mitochondria are exposed to digitonin, at detergent:protein ratios from 0.1 to 0.25 (Hovius *et al.*, 1990). As shown in Figure 6, the increase in digitonin was accompanied by increasing amounts of both ARL2 and BART in the soluble fraction. In contrast, mtHSP70 (primarily located in the matrix) and cytochrome *c* (associated with the inner membrane) were completely retained in the pellets, even at the higher concentration of digitonin. These data are representative of multiple experiments with mitochondria isolated from either sf295 cells or rat brain tissue and reveal that most of the mitochondrial ARL2 and BART was found within the intermembrane space and could be solubilized with digitonin.

Predicted Amphipathic N Terminus of ARL2 Is not Myristoylated

The discovery that ARL2 and BART can enter mitochondria prompted analysis of both proteins for potential import sequences. ARL2, like all members of the ARF family, contains an N-terminal domain that is predicted to form an amphipathic α-helix. A common feature of proteins imported into the mitochondrial matrix is an N terminus that contains several positively charged residues and is predicted

to form an amphipathic α-helix (von Heijne, 1986). Every member of the ARF family, including ARL2, has a glycine at position 2 and each ARF tested to date, including ARL1, has been found to be cotranslationally, covalently modified by the addition of myristic acid (Kahn *et al.*, 1988; Randazzo and Kahn, 1995; Lee *et al.*, 1997). The presence of this moiety at the N terminus would probably present problems in import, so we tested the possibility that cytosolic and mitochondrial pools of ARL2 may contain different modifications to their N termini.

ARL2 was examined in two different systems for N-myristoylation competence: in bacteria expressing yeast or human NMT and in cultured mammalian cells. In the former system, used previously to demonstrate acylation of other ARF family proteins (Randazzo and Kahn, 1995), bacterial cells expressing ARF1 or ARL2 were grown in the presence of [^{3}H]myristic acid and one of several NMT proteins: yeast NMT (Y), human NMT1 (H1), human NMT2 (H2), and human NMT1 containing an 81-residue amino-terminal deletion (Δ; Van Valkenburgh and Kahn, 2001). Proteins were resolved by gel electrophoresis, and the incorporation of radioactive myristate was visualized by fluorography of dried gels. Yeast and human ARF1 served as positive controls in this study and were clearly myristoylated, as indicated by labeled bands at the predicted M_r of 21 kDa, whereas the yeast [G2A]ARF1 mutant served as a negative control (Figure 7A). No incorporation of [^{3}H]myristate into ARL2 could be detected using this method, even with longer exposure times, despite its expression to comparable levels as controls (Figure 7A).

Human ARFs are inefficiently modified in the bacterial system, so we also monitored the incorporation of [^{3}H]myristate into ARL2 in mammalian cells expressing endogenous NMTs. CHO cells were transiently transfected with plasmids directing the expression of C-terminal myc-tagged human ARF5 or ARL2, and N-myristoylation was monitored after metabolic labeling with [^{3}H]myristate, as described under MATERIALS AND METHODS. A labeled band of the expected M_r was detected in cells expressing ARF5 (Figure 7B). In spite of the higher levels of ARL2 expression achieved in these cells, there was no detectible labeling of the immunoprecipitated protein (Figure 7B). We conclude that, in contrast to every other member of the ARF family tested previously, human ARL2 is not N-myristoylated. In related studies, we found that human ARL3 is also not N-myristoylated (our unpublished data). The lack of myristoylation of ARL2 eliminates this potential barrier to import, and thus no differences in covalent modifications between cytosolic and mitochondrial ARL2 could be discerned.

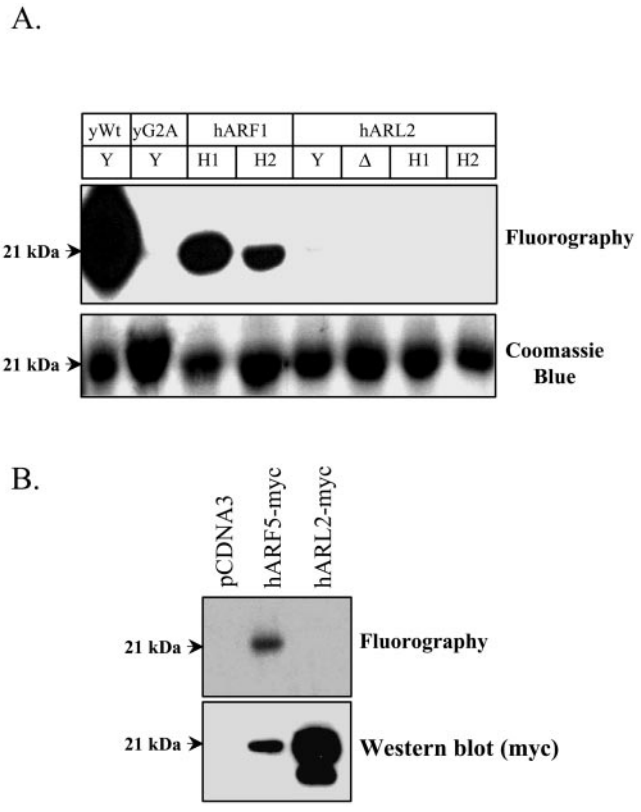

Figure 7. ARL2 is not N-myristoylated in cultured mammalian cells or in bacteria expressing N-myristoyltransferase. (A) Covalent incorporation of [^{3}H]myristate into human ARL2 was monitored in bacteria coexpressing *S. cerevisiae* (Y) NMT, human NMT1 (H1), human NMT2 (H2), or the N-terminally truncated form of human NMT1 (Δ) together with human ARL2 (hARL2), human ARF1 (hARF1), yeast ARF1 (yWT), or yeast [G2A]ARF1 (yG2A), as described under MATERIALS AND METHODS. Cell lysates were resolved in SDS gels and stained with Coomassie blue (bottom) before fluorography (top). The yeast and human ARF proteins are included as positive controls and the [G2A]ARF1 mutant as a negative control. Note the complete lack of myristate incorporated into human ARL2, despite its being expressed to similar levels as positive controls. (B) Empty vector control (pcDNA3), C-terminally tagged human ARF5 (hARF5-myc), or C-terminally tagged human ARL2 (hARL2-myc) were transiently transfected into CHO cells in the presence of [^{3}H]myristic acid, as described under MATERIALS AND METHODS. After 16 h, expressed proteins were isolated by immunoprecipitation and resolved in SDS gels before fluorographic (top) and immunoblot detection (bottom). Note the lack of label in the ARL2 expressing cells, despite the higher levels of protein expressed relative to the ARF5 control.

ARL2·GTP·BART Complex Binds Bovine ANT

The high affinity of BART for ARL2·GTP (≤20 nM) made it possible to screen for proteins that bound the complex, but not ARL2·GTP alone, by using a modification of the gel overlay assay. Little or no binding of the ARL2·[α-^{32}P]GTP·BART complex could be detected in the gel overlay assay when whole cell lysates were probed. With knowledge of the presence of ARL2 and BART in mitochondria, bovine brain fractions enriched for mitochondria were assayed and a protein of 32 kDa was found to bind the complex specifically (Figure 8). Binding activity was also evident in mitochondria isolated from rat brain tissue and sf295 cells. No activity was detected when the probe was [α-^{32}P]GTP alone or ARL2·[α-^{32}P]GTP (Figure 8). Thus, using the overlay assay, we were able to detect the presence of a specific binding partner in mitochondria for the ARL2·GTP·BART complex.

Initial characterization of the 32-kDa protein revealed a tight association with the membrane fraction after chemical or physical disruption of mitochondria. Sequential extractions with detergents and/or alkaline carbonate allowed the partial purification of this activity. It quickly became apparent that the binding activity copurified with a relatively

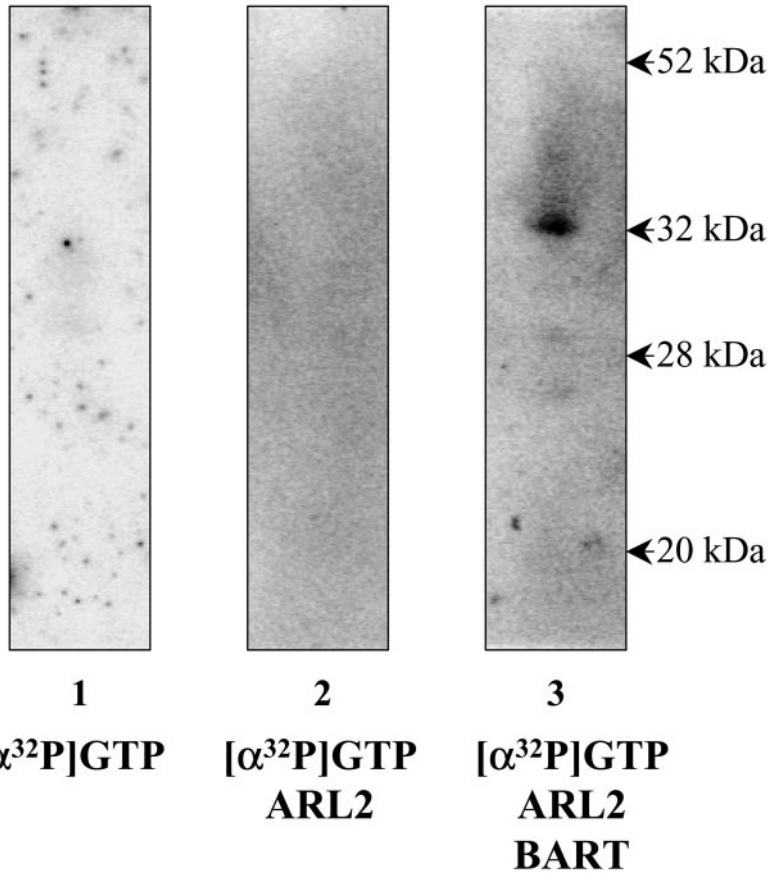

Figure 8. ARL2·GTP·BART complex specifically binds a 32-kDa protein that is enriched in mitochondria. Rat brain mitochondria (40 μg/lane) were resolved by gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Filter-bound proteins were then subjected to overlay analysis with either [α-^{32}P]GTP alone (lane 1), ARL2 prebound to [α-^{32}P]GTP (lane 2), or ARL2·[α-^{32}P]GTP·BART (lane 3), as described under MATERIALS AND METHODS. Radioactivity was detected with a PhosphorImager after 3–6-h exposure. Similar results were obtained in multiple independent experiments.

abundant 32-kDa protein band, and extended carbonate extraction alone was sufficient for isolation of the binding protein as a single band in that region of the polyacrylamide gel. Bovine brain mitochondria were treated with alkaline carbonate and the resulting insoluble material was collected by centrifugation and resolved by gel electrophoresis. Approximately 40 pmol of the 32-kDa protein was excised from the gel, digested with trypsin, and subjected to Q-TOF MS/MS analysis to determine sequences of the derived peptides. Five such peptide sequences were obtained in this manner and each was present within one polypeptide in the protein database, bovine adenine nucleotide transporter 3 (ANT3; Figure 9).

```
1   MTEQAISFAK DFLAGGIAAA ISKTAVAPIE RVKLLLQVQH ASKQIAADKQ YKGIVDCIVR
61  IPKEQGVLSF WRGNLANVIR YFPTQALNFA FKDKYKQIFL GGVDKRTQFW RYFAGNLASG
121 GAAGATSLCF VYPLDFARTR LAADVGKSGS EREFRGLGDC LVKITKSDGI RGLYQGFNVS
181 VQGIIIYRAA YFGIYDTAKG MLPDPKNTHI VVSWMIAQTV TAVAGVVSYP FDTVRRRMMM
241 QSGRKGADIM YKGTVDCWRK ILKDEGGKAF FKGAWSNVLR GMGGAFVLVL YDELKKVI
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Figure 9. Microsequencing of the purified 32-kDa binding protein identified five peptides from bovine ANT3. The five tryptic peptide sequences obtained from analysis of the 32-kDa binding protein sample are shown by underlining within the complete sequence of bovine ANT3 (accession number P32007).

ARL2·GTP·BART Binds Mouse ANT1 but not ANT2

Nucleotide transporters are abundant proteins in mitochondria, so it was especially important to confirm the identity of the ARL2·BART binding protein. The availability of transgenic mice deleted for ANT1 (Graham *et al.*, 1997) allowed for definitive analysis of binding to different rodent ANT species. Although humans and cows express three ANT isoforms, rodents contain only two. Thus, the *ant1*$^{-}$*/ant1*$^{-}$ mice also afforded an opportunity to assess specificity among isoforms. The two murine ANT proteins differ in tissue expression and abundance (Graham *et al.*, 1997), with ANT1 expressed in heart, skeletal muscle, and brain (Graham *et al.*, 1997; Figure 10, top), and ANT2 found in all tissues (Graham *et al.*, 1997; Figure 10, bottom). As expected, no ANT1 protein was detected in mitochondria isolated from tissues obtained from the transgenic animals (Figure 10, top, right). When the same mitochondrial preparations were assayed for the binding of the ARL2·BART complex (Figure 10, middle), activity was present in tissues from the wild-type animal, and the amount of activity paralleled the immunoreactive material seen in the ANT1 blot. In contrast, no activity was detected in mitochondria isolated from the same tissues of *ant1*$^{-}$*/ant1*$^{-}$ animals (Figure 10, middle). These results confirmed the identification of ANT1 as a binder of the ARL2·BART complex in mouse mitochondria and support the identification of bovine ANT3 as the protein

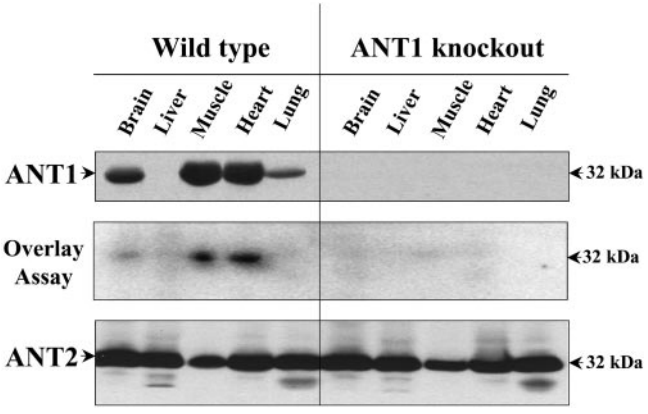

Figure 10. Murine ANT1, but not ANT2, is an ARL2·BART binding protein. Mitochondria were purified from the indicated tissues of wild-type (*ant1*$^{+}$/*ant1*$^{+}$) or *ant1*$^{-}$/*ant1*$^{-}$ mice and assayed by immunoblot and gel overlay analyses. Immunoblots were performed with ANT1 (top) or ANT2 (bottom) antibodies and the overlay assay used ARL2·[α-^{32}P]GTP·BART as probe. The lack of ANT1 immunoreactivity and activity in the binding assay are evident in the mitochondria from the knockout (KO) animal (right). These results are representative of multiple independent experiments.

with the same activity in bovine brain mitochondria. Interestingly, ANT2 is the most abundant ANT isoform in several other tissues, with levels comparable to ANT1 in heart and brain, and yet no binding activity was ever seen in the absence of ANT1. Thus, the ARL2·BART complex exhibits specificity for the binding of the ANT1 isoform in mice.

BART Can Bind to ANT Independently of ARL2

In the overlay assay, the radioactive label required for signal detection is bound to ARL2. Therefore, this method could not be used to assay for direct binding of BART to ANT. We modified the technique to allow direct tests of the ability of BART alone to bind ANTs by generating radiolabeled BART, by using in vitro translation in the presence of [^{35}S]methionine and cysteine. In this way we were able to demonstrate that BART was capable of interacting with ANT1 in the absence of ARL2 (Figure 11). The interaction of in vitro-translated BART with murine ANT1 was confirmed by comparing activities in mitochondria isolated from wild-type and transgenic animals (Figure 11, lanes 5 and 6). The 32-kDa band was detected when radiolabeled BART was present in the assay (Figure 11, lanes 2, 3, and 5) but not with comparable levels of radiolabeled ARL2 alone (Figure 11, lane 1). Excess unlabeled BART effectively competed the binding of [^{35}S]BART to ANT (Figure 11, lane 4). The presence of an ~20-kDa protein was often, although not always (Figure 11, compare lanes 2 and 3 with lanes 5 and 6), seen in these assays, particularly when radioactive BART was used. Originally thought to be endogenous ARL2, the interaction is not dependent on added GTP and is under investigation.

Intramitochondrial Levels of ARL2 Are Increased in Mice Deleted of ANT1

The expression of a number of proteins is known to be increased in mitochondria isolated from *ant1*$^{-}$/*ant1*$^{-}$ mice (Esposito *et al.*, 1999; Murdock *et al.*, 1999), presumably as part of a compensatory response to the loss of the translocase. Although some of these are directly involved in oxidative phosphorylation (e.g., components of complex I and

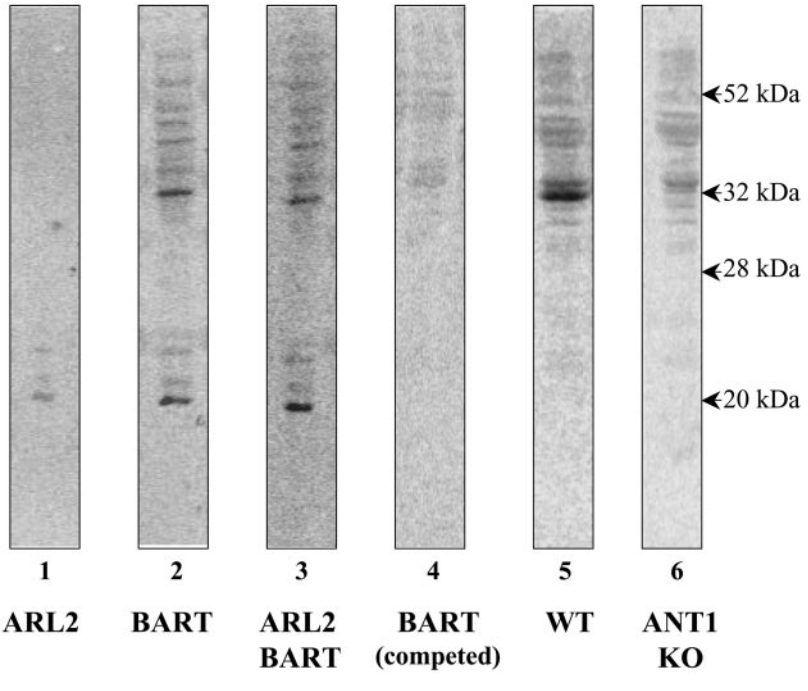

Figure 11. BART can bind ANT1 independently from ARL2. [^{35}S]-labeled ARL2 and BART were prepared in separate transcription/translation reactions and used, either alone or in combination, in overlay assays of rat (lanes 1–4) or mouse (lanes 5 and 6) brain mitochondria as described under MATERIALS AND METHODS. No signal was detected in the region of the 32-kDa binding protein when using ARL2 alone (lane 1), but signal was clearly evident when using either BART alone (lane 2) or BART in combination with ARL2 in the presence of GTP (lane 3). This activity was effectively competed with unlabeled, recombinant BART (20 μM; lane 4) and was not observed in the ANT1 knockout (KO) mouse (lane 6). The presence of the doublet (most evident in lane 5) has been noted before (Wallace, unpublished observation) and results from effects of Laemmli's sample buffer, probably SDS, on ANT. This experiment was performed at least three times with essentially identical results.

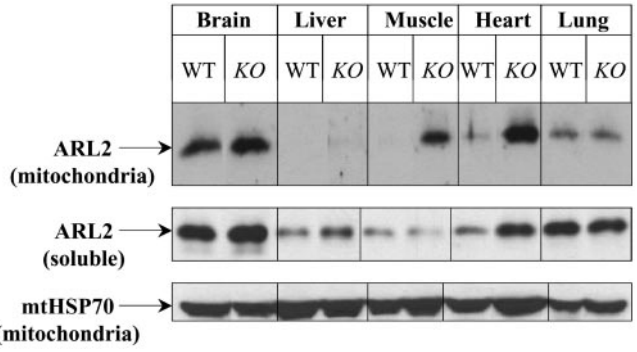

Figure 12. Amount of ARL2 in mitochondria from skeletal muscle and heart is markedly increased in animals deleted for ANT1. Mitochondria (top and bottom) and total soluble protein (S100; middle) from the indicated tissues were isolated from parental or *ant1*$^{-}$/*ant1*$^{-}$ mice and analyzed by immunoblotting (25 μg of protein/lane) by using ARL2 (top and middle) or mtHSP70 (bottom) antibodies. Differences in ARL2 levels inside mitochondria, compared with S100, are evident from comparisons of the top and middle panels. This experiment was repeated using tissues isolated from four separate pairs of control and knockout animals, with essentially identical results.

IV, malate dehydrogenase, and glycogen phosphorylase; Esposito *et al.*, 1999; Murdock *et al.*, 1999), others had not previously been linked with mitochondrial metabolism (e.g., Mcl-I, WS-3, and Skd3; Esposito *et al.*, 1999). To determine the impact of the loss of ANT1 on total or intramitochondrial levels of ARL2 and/or BART, both total soluble protein and purified mitochondria were isolated from *ant1*$^{-}$/*ant1*$^{-}$ and control mouse tissues and analyzed by immunoblot along with mtHSP70, which served as a control for gel loading and protein preparations. Total and mitochondrial levels of BART (our unpublished data) or mtHSP70 (Figure 12) were virtually unchanged in all five tissues examined. Of the five mouse tissues analyzed, only the heart showed increased levels of soluble ARL2 in knockout versus control animals (Figure 12, middle). A much more dramatic increase was evident in the levels of heart mitochondrial ARL2 and was also seen in skeletal muscle mitochondria (Figure 12, top). Indeed, ARL2 was virtually undetectable in wild-type skeletal muscle mitochondria but approached levels seen in brain or heart in the knockout animals. Identical results were obtained with four separate pairs of parental and transgenic animals. The constancy of soluble ARL2 levels in skeletal muscle supports the conclusion that import into mitochondria is under some form of regulation that is specifically sensitive to the absence of ANT1, at least in this tissue, but probably also heart.

DISCUSSION

Our results document the presence within mitochondria of a subpopulation of cellular ARL2 and its binding partner, BART, and that ARL2·GTP·BART, or BART alone, binds directly and specifically to certain isoforms of the adenine nucleotide transporter in in vitro assays. Mitochondria from the two muscle tissues were found to have substantially increased levels of ARL2, but not BART, in response to the deletion of ANT1. These observations provide initial support for the hypothesis that ARL2 and BART are imported into mitochondria in a regulated manner and there may bind to ANT1 (rodent) or ANT3 (bovine) to regulate adenylate translocase and mitochondrial function(s).

Our conclusion that subpopulations of ARL2 and BART reside within mitochondria is based on the following observations. 1) Indirect immunofluorescence, using antibodies specific to each (endogenous) protein or epitope-tagged, overexpressed protein revealed clear evidence of colocalization with mitochondrial markers in cultured mammalian cells. 2) Immunostaining of mitochondria with ARL2 or BART antisera requires permeabilization of mitochondrial membranes with Triton X-100 and is not seen when only the plasma membrane is permeabilized, with saponin. 3) Both ARL2 and BART are reproducibly detected in fractions defined as being enriched for mitochondria by using previously established cellular fractionation and organelle isolation methods. In particular, the data shown in Figure 4 demonstrate colocalization of ARL2 and BART with mtHSP70 after isolation of heavy mitochondria and isoosmotic density gradient centrifugation. 4) ARL2, BART, and markers for intramitochondrial proteins in fractions enriched for mitochondria are each protected from protease digestion, whereas the ARL2 and BART in cytosol are completely degraded under identical conditions. In contrast, all other members of the ARF family are absent in enriched mitochondria preparations and bind to membranes in a rapidly reversible manner, making them sensitive to proteases. 5) Solubilization of the outer mitochondrial membrane with digitonin releases the bulk of the mitochondria-associated ARL2 and BART. Unfortunately, our antisera were not useful for immunolocalization at the level of electron microscopy.

Most proteins that are targeted to the mitochondrial matrix contain an amino-terminal presequence, generally 20–60 residues in length, that is predicted to form an amphipathic α-helix (von Heijne, 1986). Members of the ARF family have N-terminal amphipathic α-helix and along with the covalently bound myristate form the guanine nucleotide-sensitive "myristoyl switch" (Buser *et al.*, 1994; Randazzo *et al.*, 1995; Tanaka *et al.*, 1995; Ames *et al.*, 1996). Structures have been solved for four different members of the ARF family and each reveals the presence of an amphipathic α-helix at the N terminus (Amor *et al.*, 1994; Goldberg, 1998; Menetrey *et al.*, 2000; Amor *et al.*, 2001). ARL2 is unusual (among members of the ARF family) in that it is not N-myristoylated either when coexpressed in bacteria with N-myristoyltransferases, or in cultured mammalian cells (Figure 7). The lack of myristate on ARL2 may be a key to the specificity with which it gets targeted to mitochondria. The need for additional signals for mitochondrial import is suggested by the findings that the only other member of the ARF family identified to lack myristate, ARL3, was not found associated with mitochondria and only a fraction of total cellular ARL2 was found inside mitochondria. In contrast to our assumption that N-myristoylation may be inhibitory to entry of ARL2, a role for N-myristoylation in mitochondrial targeting or activation of activities is suggested by the work of Zha *et al.* (2000).

The majority of the mitochondria-associated ARL2 and BART is solubilized when the outer membrane is progres-

sively extracted with increasing concentrations of digitonin. In contrast, both cytochrome *c* and mtHSP70 remained associated with the mitoplasts after digitonin treatments. The simplest interpretation of these results is that ARL2 and BART are soluble proteins in the intermembrane space, although reversible, functional association(s) with either the inner or outer membrane is likely. Another possibility for the incomplete solubilization of ARL2 and BART is that they could bind to contact points, like the (undefined) GTP-binding proteins detected within mitochondria (Thomson, 1998).

Data from studies in the mouse revealed that ARL2·BART bound ANT1 in vitro but not ANT2, whereas bovine ANT3 was clearly present in the purified binding protein preparation. Because ANT1 and ANT3 are each expressed in cow brain and have very similar molecular weights it is likely that each is present in the protein preparation that was sequenced. Indeed, three of the five peptides obtained corresponded to regions of the proteins that were identical. The presence of three amino acid differences between ANT1 and ANT3 in peptide 1 and one difference in peptide 3 definitively identified ANT3 as being present in this preparation. Thus, in contrast to murine ANT1 we lack confirmatory evidence for the binding of ARL2·BART to bovine ANT3. The specificity of ARL2·BART or BART alone for rodent ANT1, but not rodent ANT2, has potential implications regarding the functional specificities of the different ANT isoforms. Specific binding of ARL2·BART to ANT1 was suggested by the fact that ANT2 levels in mitochondria from brain, liver, and lung are comparable to levels of ANT1 in heart, skeletal muscle, and brain (Stepien *et al.*, 1992), yet binding activity correlated with ANT1 expression and no activity was observed in mitochondria from animals deleted for ANT1. We conclude that neither BART nor ARL2·BART bind to ANT2 in the overlay assay.

BART alone can interact with ANT, independently of ARL2, and a complex consisting of ARL2, BART and ANT can also form, at least in vitro. This suggests that BART can bind both ARL2 and ANT simultaneously, although we cannot yet determine whether binding to one protein alters affinity for the other. We have been unable to document the binding of ARL2/BART to ANT1 by using independent techniques (including native blue gels and coimmunoprecipitation after overexpression of myc-ANT1; our unpublished data) or to document functional effects of the binding to ANT1 on its activity. Thus, although ARL2 and BART clearly associate with mitochondria in a specific manner, the hypothesis that the interaction with ANT1 is a functional one requires further experimental confirmation. We speculate that ARL2 and BART are part of a mechanism sensitive to changes in cytoplasmic conditions associated with ANT function (e.g., levels of ATP/ADP, reactive oxygen species, or pH) and may comprise part of a signal transduction pathway for modulating one or more ANT activities. Lack of ANT1 results in metabolic acidosis and a severe defect in coupled respiration associated with lack of mitochondrial ATP (Graham *et al.*, 1997). Preliminary data suggest that ARL2 and BART staining of sf295 mitochondria is altered under conditions of ATP depletion due to either oxygen deprivation or inhibition of both electron transport and glycolysis (Sharer and Kahn, unpublished observations). Further studies directed toward determining the relationship between cellular ATP requirements and ARL2/BART interaction with ANT are underway. Preliminary data in sf295 cells also indicate that ARL2 and BART are released from mitochondria in response to the inducer of apoptosis, atractyloside. However, no signaling role is envisioned for ARL2 or BART, comparable with that of cytochrome *c*, due to the constant presence of each protein in cytosol.

Intramitochondrial levels of ARL2, but not BART, are increased markedly in heart and skeletal muscle mitochondria obtained from animals deleted for ANT1. This finding could in theory reflect increased import of ARL2, decreased degradation in mitochondria, or a decrease in a potential protein export process. It is curious that BART levels do not change in the absence of ANT1, because this is one of the few instances in which ARL2 and BART clearly differ. The change in intramitochondrial ARL2 levels implicates a regulated process tied to ANT1 function in mice.

The presence of only a fraction of total cellular ARL2 or BART in mitochondria leaves open the possibility for other actions by each of these proteins in cytosol or other organelles. It is unlikely that the soluble ARL2 and BART are present only as depots for recruitment into mitochondria. A role for ARL2 in microtubule folding and dynamics has been proposed recently, based upon its binding to cofactor D (Bhamidipati *et al.*, 2000). Because ARL2 was shown to bind cofactor D only in the GDP bound state it is tempting to speculate that activation of ARL2 would lead to dissociation from cofactor D and import into mitochondria. Thus, tubulin dynamics and import of ARL2 into mitochondria, with consequent changes in ANT function, may be linked. Finally, IκB-α, the inhibitory subunit of the regulator of immune and inflammatory responses nuclear factor-κB, has also recently been shown to interact with ANT proteins (Bottero *et al.*, 2001) and is similarly maintained in both cytosolic and mitochondrial pools. Thus, signaling between nuclear or cytosolic proteins/functions and those inside mitochondria is likely to be more extensive and dynamic than previously appreciated.

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