Cytosolic Arl2 Is Complexed with Cofactor D and Protein Phosphatase 2A*

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Arl2 is a member of the ADP-ribosylation factor family of 20-kDa GTPases that is highly conserved in eukaryotes. Recent results revealed that a portion of cellular Arl2 and its binding partner, BART, localize to mitochondria. Because ~90% of cellular Arl2 is cytosolic, we investigated properties of the soluble protein and found that it is stably bound in a complex that migrates in gel filtration medium with a predicted molecular mass of ~300 kDa. This complex was purified ~500-fold from the soluble fraction of bovine brain. Protein components were identified by mass spectroscopy and revealed the presence of four other proteins that include the tubulin folding cochaperone cofactor D and all three subunits of at least two protein phosphatase 2A (PP2A) protein phosphatase trimers. The presence of more than one PP2A B-type subunit and the low stoichiometry of Arl2 indicate that the purified preparation still contains a mixture of complexes that cannot currently be completely resolved. Thus, although all the soluble Arl2 in bovine brain is in high molecular mass complexes, only a portion of the total cellular cofactor D and PP2A are associated with the Arl2. We further show that the Arl2 in the complex cannot bind GTP and that complexed cofactor D does not efficiently participate in tubulin refolding reactions in a manner comparable with free cofactor D. Our data suggest functional roles for the cytosolic Arl2 complex in modulating tubulin and microtubule behavior as well as a possible role in apoptosis.

The ADP-ribosylation factor (Afr) family of monomeric, regulatory GTPases is comprised of as many as 23 members in mammals and can be divided into the Afr and Afr-like (Arl) proteins. Although the Arfs share a number of conserved activities and ~60% sequence identity, the Arls are more divergent in both sequence (40–60% identity to each other or to any Afr) and function. For example, Arl2 shares 45% identity to Arl1 yet lacks each of the biochemical activities previously ascribed to Arfs.

Arl2 is notable for a number of unusual features within the Arf family, including the apparent lack of N-terminal myristoylation, rapid and phospholipid-independent binding of GTP, and association with mitochondria (1, 2). The first Arl2 effector described, BART ( binder of Arl2; 20 kDa) (3), binds to Arl2-GTP with high affinity ($K_D = 20 \text{ nM}$) and also partially localizes to mitochondria. The BART-Arl2-GTP complex can bind the inner mitochondrial membrane protein, adenine nucleotide transporter 1, in vitro assays (1). Other binding partners for activated Arl2 have been described (PDEδ and HRG4) and, like BART, shown also to bind activated Arl3 (4–7). Only 5–10% of bovine brain Arl2 fractionated with mitochondria, and the rest appeared in the cytosol (100,000 × g supernatant). In contrast to these data implicating a role for Arl2 (and BART) in mitochondrial function(s), a number of genetic studies point to roles for Arl2 in microtubule dynamics.

The Arl2 ortholog in the yeast Saccharomyces cerevisiae, Cin4p, is encoded by one of three nonessential genes isolated from screens aimed at identifying mutations with altered responsiveness to microtubule-directed stress (cold or benomyl) (8). Later, all three genes were shown to be involved in microtubule stability (9). A very similar story emerged from studies of Schizosaccharomyces pombe, in which the Arl2 ortholog, Alp41, was identified as an essential gene required for microtubule dynamics and the establishment of growth polarity (10). This screen also identified orthologs of the tubulin folding chaperones cofactor D (Alp1) and E (Alp21).

Tubulin folding and the formation of polymerization-competent αβ tubulin heterodimers is a complex process that requires the sequential actions of the 800-kDa cytosolic chaperonin CCT (11), followed by interactions with five tubulin-specific chaperones or cofactors, termed cofactors A–E (reviewed in Ref. 12). Purified cofactors C–E participate in tubulin heterodimer formation through a step that involves the hydrolysis of GTP by β-tubulin (13). The hydrolysis of GTP serves as a switch for the release of the native tubulin heterodimer from a supercomplex containing cofactors C–E and α- and β-tubulin. In addition, cofactors C–E can act on native tubulin heterodimers, such that the supercomplex is reformed. This cycle (termed the back-reaction) is thought to constitute a quality control mechanism that serves to continuously monitor the guanine nucleotide hydrolytic activity of β-tubulin in the heterodimer.

The biological significance of this process lies in the fact that the ability of β-tubulin to hydrolyze GTP is an essential property for the proper functioning of microtubule dynamics. The same reaction also serves to regulate microtubule dynamics by modulating the availability of GTP-bound heterodimers (14, 15). Direct, biochemical evidence for a role of Arl2 in the complex process of the regulation of the action of one of these cofactors, cofactor D, was provided by Bhamidipati et al. (16). This study...
AR-2 was described by Ogris et al. overnight. After clarifying by centrifugation at 100,000 g, the supernatant was collected, and the process was repeated in 40% (NH₄)₂SO₄. Solid (NH₄)₂SO₄ was added to S100 (500 ml) to bring the concentration to 20% and then stirred at 4 °C for 1 h. After centrifugation at 100,000 x g for 1 h, the supernatant was collected, and the protein concentration was determined using a Bio-Rad protein assay kit. The protein concentration was measured at 280 nm, and the turbidity of the solution was determined using a spectrophotometer. The protein concentration was calculated using the extinction coefficient at 280 nm for bovine serum albumin (BSA). The resulting pellet was resuspended in buffer A to a concentration of 20 mg/ml and then stored at -80 °C.

Fractions containing at least 50% peak level of Arl2 were pooled and analyzed by SDS-PAGE and western blotting. The protein band was excised from the gel and digested with trypsin. The resulting peptides were analyzed by LC-MS/MS. The protein profile was compared to the previously published genetic data implicating Arl2 in microtubule dynamics. These observations provide a biochemical basis for a role for these proteins in the regulation of microtubule dynamics that is predicted from genetic studies in several model systems.
Characterization of Arl2 Complexes

Coimmunoprecipitation of Cofactor D—Purified bovine brain Arl2 complex (100 μg) in buffer A was incubated overnight at 4 °C with 2 μg of monoclonal antibody 1D6. The immune complexes were precipitated by addition to 50 μl of pre-equilibrated protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences). The beads were then washed three times with buffer A before being resuspended in sample buffer (35) and analyzed by immunoblot.

Nucleotide Binding of Complexed Arl2—Binding of radionucleotides ([3H]GDP or [35S]GTPγS) to Arl2 was determined as previously described (3), using the filter trapping method to separate bound and free nucleotides. Binding of nucleotides to Arl2 in the complex was compared with that of purified recombinant Arl2. Bacterially expressed human Arl2 was purified as previously described (3).

Tubulin Refolding Assays—Tubulin refolding assays were performed and analyzed as described by Bartolini et al. (36).

Protein Phosphatase Assay—A serine/threonine phosphatase assay system (Promega) was used to determine the specific activities for P2A-containing complexes, according to the manufacturer’s specifications. The reaction was allowed to proceed for 15 min at 37 °C. The optical density of products was determined using a plate reader to read the absorbance at 600 nm. The samples were compared with a standard curve of 0–4000 pmol of free phosphate.

RESULTS

Cytosolic Arl2 Is Present in a High Molecular Mass Complex—Arl2 and its binding partner, BART, were previously shown to localize to mitochondria (1). In that study it was noted that only a small fraction (estimated at 5–10%) of total cellular Arl2 was found to be associated with mitochondria, with the remainder appearing in the supernatant after centrifugation at 100,000 × g (100S) and thus assumed to be cytosolic. As an initial test of the properties of this soluble pool of Arl2, the 100S fraction from bovine brain was fractionated on a Superdex 200 gel filtration column. Immunoblot assays revealed that Arl2 eluted as a single peak. Surprisingly, comparison of the migration of Arl2 to protein standards indicated that the Arl2 emerged from the column with an apparent molecular mass of ~300 kDa (Fig. 1). In contrast, the cytosolic BART migrated in a manner consistent with a monomeric 20-kDa protein. Purified, recombinant Arl2 has previously been found to migrate as a monomer in a number of different gel filtration media, suggesting that the high molecular mass species in which Arl2 is present in cytosol is not simply a homo-oligomeric form of Arl2.

In addition, all other Arf family members, including the most closely related protein, Arl3 (data not shown), each migrate as monomeric species in gel filtration chromatography. The nature of this high molecular mass complex was therefore investigated further.

The presence of Arl2 in a high molecular mass complex was also evaluated in S100 from a number of different tissue sources, including bovine liver and retina, rat brain and liver, and a panel of tissue culture lines, including COS-7, Sf295, HeLa, and normal rat kidney cells. In each case, the Arl2 was found exclusively in a complex that eluted in the same fractions as that of bovine brain S100 Arl2, shown in Fig. 1. Because it is the most abundant source of Arl2, brain was the source for most of the purifications performed. Purifications of the Arl2 complex were performed from bovine liver and retina and yielded results similar to those described below for bovine brain, although each was less pure than the final brain preparation.

Purification of the Cytosolic Arl2 Complex—The fact that essentially all soluble Arl2 from bovine brain was present in the complex and that the brain extract could be stored on ice for at least a week with no change in the chromatographic behavior of the Arl2 suggested that the complex in cytosol was quite stable. No appreciable dissociation of Arl2 into monomer was found at any step during the purification. Despite the fact that Arl2 is most abundant in brain, at ~0.0001% of soluble protein (estimated by immunoblot assay), Arl2 is much less abundant than Arfs (~0.01–0.3% of total cell protein) and has never before been purified from any tissue. The cytosol from one calf brain contains only about 2 μg of Arl2 (Table I) and is most readily detected by immunoblot assays.

Soluble Arl2 was purified from bovine brain by sequential chromatography after ammonium sulfate fractionation, using anion exchange, hydroxyapatite, cation exchange, and gel filtration media (see “Materials and Methods”). The purification scheme is summarized in Table I; the scheme allowed the purification of less than 1 mg of complex with about 10% recovery of Arl2 and resulted in a ~500-fold increase in specific activity of Arl2. Note that immunoblotting was the sole assay used to locate the Arl2 throughout the purification process. Specific activity is defined as the amount of Arl2, determined by comparative immunoblotting using purified recombinant Arl2 as standard, divided by total protein. The recovery of Arl2 is consistent with losses resulting from pooling of peak fractions over multiple steps, although we cannot exclude the possibility of undetectable amounts of Arl2 being lost by dissociation from the complex.

The protein profile from the peak fractions of the final (gel filtration) step in a preparation is shown in Fig. 2. Four proteins were found always to be present in the most highly enriched fractions and to have elution profiles essentially identical to that of Arl2. These four proteins also represent the most intensely staining components in the preparation and have apparent molecular masses of 130, 63, 56 (doublet), and 36 (doublet) kDa. The differences in migration of the two ~56- and ~36-kDa bands, referred to as doublets, varied between gels and preparations. The presence of an additional band, also often a doublet, at ~43 kDa in SDS gels (seen faintly in Fig. 2) was often but not always observed. Its absence from some preparations and lower staining intensity in all indicate that it is not an obligate component in the complex (see below). Analysis of each obligate component in the Arl2 complex is presented below.

Cofactor D—The protein migrating in SDS gels with an
Characterization of Arl2 Complexes

estimated molecular mass of ~130 kDa was predicted to be the tubulin-specific chaperone, cofactor D, based upon its previously described binding to Arl2 (16). A cDNA encoding bovine cofactor D has been cloned and sequenced (accession number T18522) and predicts a protein product of 1199 amino acids with a molecular mass of 133 kDa. Rabbit polyclonal antibodies to bovine cofactor D were used in immunoblots and confirmed the presence of cofactor D in our purified preparation of the Arl2 complex. Furthermore, Arl2 and cofactor D immunoreactivities had identical elution profiles in the final step of the purification of the complex (Fig. 3), and the intensity of signal in immunoblots always matched the intensity of dye binding of the ~130-kDa band in SDS gels.

PP2A—the most intensely staining (with Coomassie Blue or colloidal Coomassie stain) band in each preparation of the Arl2 complex was that migrating with a predicted molecular mass of ~63 kDa in stained SDS gels. MALDI-MS/MS analysis identified 14 peptides with masses identical to those predicted from a tryptic digestion of murine PP2A-A subunit and covered >22% of the protein sequence. The only other match with a significant score from Prowl searching or to a mammalian protein was to a human keratin, a contaminant found in all of the bands analyzed. PP2A-A subunits are always found bound to the catalytic C subunits of PP2A or in a heterotrimer that also contains a regulatory B-type subunit. Mammals express two isoforms of PP2A A proteins, α and β, that are 87% identical. Ten of the 14 peptides found by MS are present in PP2A Aα but not β, and the other 4 peptides had sequences common to α and β isoforms. No PP2A Aβ-specific sequences were identified. Thus, PP2A Aα was present in the complex, but we cannot rule out the possibility that some of the β isoform was also present.

The nomenclature for this family of proteins is confusing, but the A subunits (also termed PR65 or R1) are thought to serve as scaffolds that coordinate the binding of the catalytic C subunit with the much more diverse, regulatory B-type subunits. The human PP2A Aα protein (accession number P30153) is 589 residues in length and has been reported to migrate in SDS gels with an apparent molecular mass of between 61 and 65 kDa. Monoclonal antibody 4G7, specific to PP2A A subunit and reactive against the bovine protein, was used in immunoblots and confirmed the presence of this protein in the 63-kDa band excised from gels and its coelution with Arl2 in the gel filtration column at the end of the Arl2 complex preparation (Fig. 3).

Protein(s) with apparent molecular mass of ~56 kDa were also evident in the complex. This band actually appeared as a doublet, often more closely spaced than shown in Fig. 2. The preparation used for protein identification contained a very closely spaced doublet that was analyzed by MALDI time-of-flight MS, resulting in the identification of 15 peptides (accounting for 29% of the protein) with masses identical to those found in human PP2A B′e subunit (accession number Q16537; also termed B56e, PR61e, and R5e). Comparison of predicted peptides to members of the B′ family revealed that 10 of 15 peptides were unique to PP2A B′e, five were common to the ε isoform plus at least one other isoform, and none were unique to any of the other isoforms (α, β, γ, or δ). PP2A B′e is predicted to be 467 residues in length. Because of the complexity in PP2A B-type subunit proteins, this same sample was also analyzed by the more sensitive technique of nano liquid chromatography-electrospray ionization (Q-Star) MS/MS. This technique confirmed the presence of PP2A B′e by identifying seven of the same peptides seen previously plus an additional peptide. Five of these eight peptides were unique to PP2A B′e, and none were specific to any other isoform.

The electrospray ionization-MS data from the 56-kDa dou-
The protein migrating with a predicted molecular mass of 35 kDa was identified as the catalytic (C) subunit of PP2A, on the basis of 10 peptides found by MALDI-MS analysis and the post-source decay sequencing of three of those peptides. Nine of the ten peptides sequences were common to both the PP2A C and Cβ proteins, but one was unique to PP2A Cβ. The human ortholog of PP2A Cβ (accession number P11082) is 309 amino acids long. Monoclonal antibody 1D6, specific to PP2A-C, confirmed the identity of the ~35-kDa band in our preparation (Fig. 3) as a C subunit, likely Cβ.

Based on the data described above, we conclude that the cytosolic complex contains Arl2, cofactor D, and all three subunits of a PP2A heterotrimer. The comigration of these five proteins in the final step of purification was further examined in detail (Fig. 3) and provided strong evidence for their presence in a complex. However, the presence of more than one type of B subunit clearly indicates a level of heterogeneity in the assembled ~350-kDa complex(es). It is noteworthy that the complex of cofactor D, PP2A trimer, and Arl2 has a predicted molecular mass very close to that predicted from gel filtration of S100 starting material (from multiple tissues and cell sources) and of the final, purified preparation. PP2A trimers are plentiful in the S100 starting material and migrate on gel filtration media (e.g., that shown in Fig. 1) with the predicted size of ~150 kDa. However, the heterogeneity inherent in the identification of different B subunits and issues concerning stoichiometry, particularly for Arl2 (see below), leave open the possibility that other proteins present in the preparation could bind specifically. The most likely candidate can be seen in Fig. 2 to migrate with predicted molecular masses of ~43 kDa.

**Protein Phosphatase Methylesterase-1**—We noted the common presence in our preparations of a protein with mobility in SDS gels intermediate between those of PP2A-B and PP2A-C subunits (Fig. 2). It is predicted to be a 43-kDa protein by comparison with protein standards in the SDS gel and was not present in all preparations of the Arl2 complex. MALDI-MS analysis identified this band as protein phosphatase methylesterase-1 (PME-1). This was confirmed by immunoblotting using a PME-1 specific antiserum (data not shown). The human protein (accession number NP_057231) is 386 residues in length and has previously been shown to bind inactive mutant (29) or wild type (17) PP2A and cleave the methyl group from the C terminus of the catalytic subunit. The presence of all three subunits of PP2A in the Arl2 complex suggests that PME-1 may bind specifically to the PP2A in the complex. The binding of PME-1 may also suggest that at least some of the PP2A in the complex is inactive as a phosphatase. Together, these data suggest that PME-1 may bind to the complex and copurify with it but is not an obligate part of the Arl2 complex.

**Cofactor D Can Be Specifically Coimmunoprecipitated with Antibodies to PP2A C Subunit**—Although previously published data have shown that Arl2 can bind cofactor D (16) and that PP2A trimers can bind a number of different targets (21, 38, 39), the purification of a complex containing these five components is the first evidence for direct linkage between PP2A and either cofactor D or Arl2. For this reason, additional evidence of complex formation was sought. Monoclonal antibody 1D6, directed against the PP2A C subunit, was incubated with the purified complex and then precipitated by the addition of protein G-Sepharose beads. As shown in Fig. 4, cofactor D can be specifically precipitated with the antibody to the C subunit of PP2A. This is probably an inefficient process because most antibodies to PP2A subunits do not efficiently precipitate PP2A heterotrimers and may promote the dissociation of the trimer (for examples, see Refs. 29, 40, and 41). A recent study of protein complexes in the yeast *S. cerevisiae* revealed that epitope-tagged Cdc55p, a yeast PP2A B-type subunit, specifically copurified with Cin1p, the ortholog of cofactor D (42). This is consistent with the direct binding of cofactor D to PP2A B-type subunits and may explain the less than stoichiometric coimmunoprecipitation of cofactor D with PP2A C, seen in Fig. 4.
Stoichiometry of Proteins in the Arl2 Complex—Differences among proteins in their ability to bind Coomassie Blue or other dyes make determination of stoichiometry of complex components difficult. Thus, the relative amounts of each of the five proteins in the Arl2 complex are uncertain at this time, despite the fact that the size predicted from gel filtration is most consistent with single copies of cofactor D and each PP2A subunit. However, it is evident that one component, Arl2, is highly underrepresented in the complex. Arl2 cannot be seen in Coomassie Blue-stained gels of the purified complex (Fig. 2), despite the fact that purified, recombinant human Arl2 binds the dye with an efficiency comparable with other proteins (data not shown). Quantitative immunoblotting was performed as described under “Materials and Methods” to determine the amount of Arl2 in the purified complex. If we assume the preparation is pure and the complex has a molecular mass of 300 kDa, then based upon the amount of protein loaded and the amount of Arl2 found in immunoblots, we estimate that different preparations of the purified Arl2 complex had between 0.005 and 0.05 mol Arl2/mol complex. Thus, we conclude that the purified “Arl2 complex” is actually a mixture of at least two complexes, those with and without Arl2, and that cofactor D can bind PP2A independently of Arl2.

In an effort to obtain a preparation with a higher stoichiometry of Arl2 bound, we incubated the purified complex with an excess of purified recombinant Arl2 at 30 °C for 15 min and then resolved proteins by gel filtration. However, no increase in the amount of Arl2 fractionating with the complex was observed. The same results were obtained when the incubation was performed in the presence of GDP or GTP.

**Complexed ARL2 Cannot Bind GTPγS**—The ability of Arl2 in the complex to bind guanine nucleotides was determined using radioligand binding assays, as described under “Materials and Methods.” The amount of Arl2 in a preparation of the complex was determined by quantitative immunoblots, in comparison with purified recombinant Arl2, to allow binding assays to be performed with the same concentration of Arl2, in one case free and in the other bound in the complex. The rate and extent of binding of [3H]GDP to each form of Arl2 was determined and found to be the same (Fig. 5, top panel). In contrast, complexed Arl2 is unable to bind the GTP analog, GTPγS (Fig. 5, bottom panel). Because the binding of guanine nucleotides to free Arl2 is rapid, these results are also consistent with our observations that the Arl2 complex is very stable, and there is very little dissociation of Arl2 from the complex under these conditions. These observations also agree with the conclusion of Bhamidipati et al. (16), that it is GDP-bound Arl2 that binds to cofactor D. This is the first instance in which binding to another protein(s) has resulted in the loss of GTP binding to a member of the Arf family. These data also suggest that the mechanism by which Arl2 becomes activated in cells is likely to be different from those used by other GTPases.

**Tubulin Folding Activity of Cofactor D in the Arl2 Complex**—A role for cofactor D in the biosynthesis and assembly of the tubulin heterodimer has been demonstrated previously (11–13, 32). Tubulin-specific chaperones can also be assayed for their ability to refold tubulin heterodimers by using native tubulin as substrate and monitoring the refolding of α-tubulin and binding of [α-32P]GTP to the nonexchangeable nucleotide binding site. Purified cofactors C–E participate in tubulin heterodimer formation with hydrolysis of GTP bound to β-tubulin serving as a switch for the release of the native tubulin heterodimer from a supercomplex containing cofactors C–E and α- and β-tubulin (Fig. 6, first lane). In the absence of cofactor D, even in the presence of cofactors C and E, neither of these reactions can proceed (Fig. 6, third lane). With the functions of the tubulin-specific chaperones in mind, we compared the ability of purified cofactors D and D bound in the Arl2 complex for their ability to promote tubulin refolding in vitro (Fig. 6, compare first lane with fourth through sixth lanes). The results show that only trace amounts of the tubulin present in the reaction were bound to cofactor D when this cofactor was supplied in the form of the Arl2 complex. This was the case even in reactions containing a large excess of complexed (compared with free) cofactor D. These data indicate that cofactor D is very stably bound and that its tubulin binding activity is inhibited when it exists in the Arl2 complex.

**Protein Phosphatase Activity of the Complex**—The presence of PP2A trimers in the purified complex prompted an examination of its protein phosphatase activity. Binding of B-type subunits can dramatically alter specific activity and substrate specificity of the C subunit, as can a number of allosteric modulators. We obtained a purified recombinant PP2A trimer, containing Ba, from Marc Mumby (University of Texas Southwestern Medical Center) as a positive control and compared its activity to that of the purified Arl2 complex, after normalizing
Characterization of Arl2 Complexes

40835

Fig. 6. The cofactor D/Arl2/PP2A complex cannot functionally substitute for cofactor D in tubulin refolding assays. Tubulin refolding assays were performed in the presence of [α-32P]GTP, and the reaction products were resolved on native polyacrylamide gels as described under “Materials and Methods.” The arrows indicate the location of species containing chaperonin (CCT) or tubulin-specific cofactor-containing complexes generated in these reactions. PC tubulin, tubulin prepared free from associated proteins by chromatography on phospho-cellulose; α-tub, α-tubulin; β-tub, β-tubulin; CoD, cofactor D; CoCDE/CoD, complex of cofactors C-E and tubulin. This experiment has been repeated at least twice with similar results.

The presence of Arl2 in a multisubunit complex is highly unusual for a regulatory GTPase and leads to predicted novelty in modes of regulation. The ability of Arl2 to bind GTP, but not GDP, is completely inhibited as a result of its interaction with cofactor D and PP2A. This observation leads to the prediction that Arl2 in cells is predominantly in the GDP-bound state. It also may explain why purified, recombinant Arl2 can bind GTP so avidly in the pure form, yet still serve a regulatory role in cells. Unlike Rab and Rho proteins, which are found in the cytosol bound to GDP and guanine nucleotide dissociation inhibitors, GDP exchange on Arl2 is unaffected by complex formation with PP2A and cofactor D. Thus, a guanine nucleotide exchange factor may activate Arl2 either through promotion of dissociation from the complex or by support of GTP binding with consequent (predicted) release from the complex. In either case, activation of Arl2 in cells is predicted to occur through novel mechanisms. Improving the yield and homogeneity of the Arl2 complex through recombinant techniques should allow assays of Arl2 guanine nucleotide exchange factor and are currently underway.

The role of the PP2A subunits in function(s) of the Arl2 complex may prove challenging to elucidate. If the biologically relevant substrate(s) is a component of the complex, then its identification becomes tractable. If not, the problem becomes much more difficult to solve and is further complicated by the diversity of B subunits, both in cells and in our complex. Previous reports have described both binding of PP2A to microtubules and inactivation of the phosphatase activity as a result (26–28). Whether these observations are linked to those reported here remains to be seen. We found very similar specific activities for protein phosphatases in our ~300-kDa complex(es) and in a purified, recombinant trimer. Because Arl2 is under-represented, we can make no conclusions regarding a possible effect of Arl2 on PP2A activity, but the binding of cofactor D to PP2A does not appear to ablate the activity of the latter. Preliminary data reveal that cofactor D in S100 migrates as a series of 6–8 spots in two-dimensional gels with differing isoelectric points but that the cofactor D from the purified complex or prior exposure of the S100 to a nonspecific serine/threonine protein phosphatase results in the loss of the more acidic spots in two-dimensional gels. These data are consistent with the hypothesis that cofactor D is a phosphoprotein that is a substrate for PP2A in the complex, but additional work is required to more directly test this model.

The activity of cofactor D associated with our complex is clearly inhibited. The activity of cofactor D in tubulin folding has been shown previously to be regulated at some level through its interaction with Arl2 (16). The direct interaction of Arl2 with cofactor D inhibits the ability of cofactor D to interact with and disrupt the native tubulin heterodimer in vitro (i.e., the back-reaction), although the presence of Arl2 has no detectable effect on the in vitro cofactor D-dependent assembly of the tubulin heterodimer. The presence of a complex containing cofactor D with PP2A and Arl2 suggests additional roles for protein phosphorylation and PP2A in tubulin folding and function. We infer from our results the existence of a stable complex of PP2A and cofactor D that is deficient in cofactor D activity. The existence of this and other functionally related complexes in cells is of immediate interest now to models of tubulin folding and the regulation of microtubule dynamics.

Although the most likely function of complexed Arl2 is in the regulation of tubulin heterodimer levels and microtubule dynamics, we consider here a model in which free Arl2 has an additional, distinct role in cells, potentially one related to apoptosis. Four observations appear consistent with this conclusion. We have established the presence of Arl2 in mitochondria (1) and preliminary evidence indicates that cofactor D is also present in purified bovine brain mitochondria preparations. A recent report described the presence of tubulin in mitochondria
and its interaction with the voltage-dependent anion channel (43), which has been proposed to interact with the adenine nucleotide translocase in formation of the permeability transition pore, which is active in apoptosis. Our previous results showed that Arl2-GTP and its partner, BART, bind adenine nucleotide transporter 1 in vitro (1). Work from the Ruvolo laboratory revealed that ceramic treatment of cells induced the recruitment of PP2A to mitochondria and dephosphorylation of Bcl2 (44). Interestingly, this effect appears to involve the PP2A Bo protein, one of the two B-type subunits identified in our complex. Finally, when Arl2 was overexpressed in HeLa cells, we found all the new protein in cytosol to be monomeric with no apparent increase in the amount of Arl2 in the complex. In addition, transfected cells began to exhibit extensive blebbing and cell death by ~24 h post-transfection. 2 Thus, both the cytosolic Arl2 complex and the mitochondria are potential storage reservoirs to ensure low levels of free Arl2 and protection against its lethal effects. Alternatively, Arl2 may provide a regulatory link between the control of tubulin assembly/microtubule dynamics and essential functions in mitochondria. Additional experiments are required to distinguish between these alternative models.

Successful reconstitution of the pentameric complex from purified components will very likely prove to be informative of its function in cells. It is likely that other proteins may be required. Preliminary data indicated that simply mixing the five components together in vitro did not result in the formation of a stable complex. Similarly, adding excess purified Arl2 to the purified complex did not increase the amount of Arl2 bound. From other preliminary data (see above) implicating phosphorylation of cofactor D as a regulatory event, we hypothesize that the complex we describe here is simply the most stable of a series of protein complexes that are involved in regulating tubulin levels and, either directly or indirectly, tubulin dynamics.

In summary, we have documented the existence in cytosolic extracts of a complex containing five components: Arl2, cofactor D, and a PP2A trimer. Activities of at least two of these proteins are dramatically altered as a result of their inclusion in the complex and possible covalent modifications that may result. Although the function of this complex is currently unknown, a number of observations suggest that it participates in reactions involved in the assembly and dynamics of the tubulin heterodimer and possibly in apoptotic signaling at the mitochondria.

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