ADP Ribosylation Factor-like Protein 2 (Arl2) Regulates the Interaction of Tubulin-folding Cofactor D with Native Tubulin

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Abstract. The ADP ribosylation factor-like proteins (Arls) are a family of small monomeric G proteins of unknown function. Here, we show that Arl2 interacts with the tubulin-specific chaperone protein known as cofactor D. Cofactors C, D, and E assemble the α/β-tubulin heterodimer and also interact with native tubulin, stimulating it to hydrolyze GTP and thus acting together as a β-tubulin GTPase activating protein (GAP). We find that Arl2 downregulates the tubulin GAP activity of C, D, and E, and inhibits the binding of D to native tubulin in vitro. We also find that overexpression of cofactors D or E in cultured cells results in the destruction of the tubulin heterodimer and of microtubules. Arl2 specifically prevents destruction of tubulin and microtubules by cofactor D, but not by cofactor E. We generated mutant forms of Arl2 based on the known properties of classical Ras-family mutations. Experiments using these altered forms of Arl2 in vitro and in vivo demonstrate that it is GDP-bound Arl2 that interacts with cofactor D, thereby averting tubulin and microtubule destruction. These data establish a role for Arl2 in modulating the interaction of tubulin-folding cofactors with native tubulin in vivo.

Key words: Arls • G proteins • chaperones • microtubules • cytoskeleton

Introduction

Proteins belonging to the Ras superfamily use the binding and hydrolysis of GTP as a molecular switch to regulate a wide range of cellular functions. Within this superfamily, ADP ribosylation factor (ARF)[1] proteins are defined by their ability to act as cofactors in the cholera toxin-catalyzed ADP-ribosylation of G_s, and are involved in membrane transport, maintenance of organelle integrity, and the activation of phospholipase D (Donaldson and Klausner, 1994; Nuoffer and Balch, 1994; Boman and Kahn, 1995; Moss and Vaughan, 1995). A subfamily of ARF-related proteins, termed Arls, share 40–60% amino acid sequence identity with ARF proteins, but have little or no ARF activity. The function of Arls in cellular signaling pathways is completely unknown.

Microtubules are polarized polymers of α/β tubulin heterodimers that participate in a wide range of both essential and specialized cellular functions. The dynamic behavior of microtubules is controlled by polymerization-dependent GTP hydrolysis by the β-subunit and the binding of associated proteins (Mitchison and Kirschner, 1986). The generation of new tubulin heterodimers is a multistep process involving several chaperone proteins. Nascent α- and β-tubulin chains first interact with prefoldin (Geissler et al., 1998; Vainberg et al., 1998; Hansen et al., 1999), a heterohexameric chaperone that delivers its target protein to the cytosolic chaperonin, CCT (Hartl, 1996). After one or more rounds of ATP hydrolysis by CCT, the tubulin target proteins acquire a quasinative conformational state defined by the formation of the GTP-binding pocket (Tian et al., 1995). These quasinative folding intermediates (which are not competent to form tubulin heterodimers) then interact with a series of five tubulin-specific chaperone proteins known as cofactors A–E (Lewis et al., 1997; Tian et al., 1997). Cofactors A and B bind specifically to β- and α-tubulin folding intermediates, respectively, and hand off their target molecules to cofactors D and E. These cofactor/tubulin complexes then associate to form a supercomplex containing cofactors C, D, and E, and α- and β-tubulin; GTP hydrolysis by the bound tubulin then triggers the release of native α/β-tubulin heterodimers (Lewis et al., 1997).

In addition to assembling the tubulin heterodimer during the de novo folding of tubulin, cofactors C, D, and E

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[1] *Abbreviations used in this paper:* ARF, ADP ribosylation factor; Arl, ARF-like; BS3, bis(sulfosuccinimidyl) suberate; CCT, chaperonin-containing TCP-1; GAP, GTP activating protein; GDP, guanosine diphosphate; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin.

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interact with native tubulin. First, cofactors D and E can each react in vitro with native tubulin, sequestering the β- or α-subunits, respectively. Under these circumstances, the remaining partner subunit decays to a nonnative state (Tian et al., 1997). Second, cofactors C, D, and E together influence the guanine nucleotide state of the native heterodimer, stimulating the polymerization-independent hydrolysis of GTP by β-tubulin; in this regard, they act as GTP activating proteins (GAPs; Tian et al., 1999). Here, we report that expression of cofactors D or E in transfected cultured cells destroys the tubulin heterodimer and microtubules. We show that the coexpression of wild-type Arl2 or an Arl2 mutant defective in GTP binding (but not a GTPase defective Arl2 mutant) specifically prevents the destruction of tubulin and microtubules caused by expression of cofactor D. In addition, an Arl2 variant carrying a mutation in its putative effector loop fails to bind cofactor D or rescue microtubules from destruction by exogenously expressed cofactor D. Finally, Arl2 downregulates the GAP activity of cofactors C, D, and E in vitro. These data establish a role for Arl2 in modulating the interaction of tubulin-folding cofactors with native tubulin, thereby regulating microtubule dynamics.

Materials and Methods

Plasmid Construction

pGFP-C, pGFP-D, and pGFP-E were constructed by insertion of full-length cDNAs encoding cofactors C, D, or E (Tian et al., 1996) into the plasmid pEGFP-C3 (CLONTECH Laboratories, Inc.). Human Arls were cloned (by PCR) into pET23b (Novagen) using human testes mRNA (CLONTECH Laboratories, Inc.) as template; mutant forms of Arl2 were generated by PCR and checked by DNA sequencing. For transfection assays, wild-type and mutant forms of Arl2 were cloned into the plasmid pcDNA3 (CLONTECH Laboratories, Inc.) containing an NH_2-terminal hemagglutinin (HA) tag (Mader et al., 1995) and into pEGFP-C3 (CLONTECH Laboratories, Inc.). For expression of COOH-terminally His-tagged protein, these inserts were cloned into pET23b (Novagen).

Protein Expression and Purification

Tubulin and cofactors C, D, and E were purified as described previously (Tian et al., 1996). Arl2 and Arl3 were purified from extracts of host *Escherichia coli* BL21DE3 (Arl2) or BL21DE3LysE (Arl3) cells cleared by centrifugation at 100,000 *g*. An ammonium sulfate cut (Arl2, 35–55%; Arl3, 0–35%) was dissolved in 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 20 mM Tris-HCl, pH 8.3 (Arl2) or pH 9.2 (Arl3), and applied to a Q15 anion exchange column (Amersham Pharmacia Biotech), which was developed with a linear gradient containing 0.5 M NaCl. Fractions containing Arl protein were pooled, concentrated using a Centricon 10 ultrafiltration device (Millipore), and applied to a Superdex 200 gel filtration column run in 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 20 mM Tris-HCl, pH 7.5. His-tagged proteins were purified from extracts of *E. coli* BL21(DE3) cells using Talon cobalt affinity resin (CLONTECH Laboratories, Inc.), following the manufacturer's recommended protocol.

In Vitro Translation and Binding Assays

In vitro transcription/translation of Arls was done by addition of plasmids to TNT rabbit reticulocyte lysate (Promega) containing ^{35}S-methionine (0.8 mCi/ml). Reactions were cleared of particulate material by centrifugation at 200,000 *g*, incubated at 30°C for 30 min with purified cofactor D (0.27 μM), and diluted 10-fold with PBS. In some experiments, the cleared transcription/translation reaction was applied to a 2.4-ml Superdex 200 gel filtration column (SMART System; Amersham Pharmacia Biotech) run in PBS. In other experiments, rabbit anticofactor D (Tian et al., 1996) was added at a dilution of 1:20 and the incubation continued for 1 h. Antibody-bound labeled material was recovered by reaction with agarose-bound protein A/G (Cytosignal). Purified His-tagged Arl2 and Arl2 mutant proteins (12.5 μM) were reacted with translated cofactor D as described above and isolated by binding to Talon cobalt affinity resin. In all cases, the resin-bound complexes were extensively washed with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, containing 0.05% Tween 20.

GTPase Assays

Rates of GTP hydrolysis were measured in reactions done as described (Tian et al., 1999), using γ-^{32}P–labeled GTP (specific activity, 6.0 mCi/mMol) and purified bovine brain tubulin (1.7 μM) with or without added cofactors (C, 0.40 μM; D, 0.13 μM; E, 0.26 μM) and Arl2 or 3 (0.5, 1.0, 2.0 μM).

Reaction of Cofactor D with Native Tubulin In Vitro

Purified tubulin heterodimer, ^{35}S-labeled in its β-subunit (Tian et al., 1997) at a final concentration of 0.15 μM, was incubated with cofactor D (0.45 μM) either alone or with a 5- or 15-fold molar excess (with respect to cofactor D) of purified recombinant Arl2. GST (glutathione S-transferase) was used as a control. Reaction mixtures were incubated at 30°C for 1 h, and the products resolved by electrophoresis on native polyacrylamide gels as described previously (Gao et al., 1992).

Transfection and Immunofluorescence

Cultured HeLa cells were transfected using Fugene transfection reagent (Boehringer). After 40 h, cells were fixed with 4% paraformaldehyde in PBS. Cells were stained with one or more of the following antisera: polyclonal anti-HA (Santa Cruz; 1:50); monoclonal anti–α-tubulin (1:2,000); anti–β-tubulin (1:1,000; both from Sigma Chemical Co.). In some experiments, transfected cells were incubated with 10 μM nocodazole for 1.5 h (36 h posttransfection) immediately before fixation.

Cross-linking and Immunoprecipitations from Transfected Cells

Cultured 293T cells were transfected with either pGFP-D or pGFP-E, or cotransfected with pGFP-D and pHA-Arl2. Cells were harvested 48 h posttransfection, washed with PBS, and lysed in ice-cold hypotonic buffer (50 mM sodium phosphate, pH 7.4, 10 mM NaCl, 0.1% Tween 20, and 1 mM guanosine-5′-O-(3-thiotriphosphate) [in the case of pGFP-D and pGFP-E]). A cleared extract was prepared by centrifugation at 30,000 *g*. In some experiments, proteins were cross-linked by incubation of cleared cell extracts with 0.5 mM bis(sulfosuccinimidyl) suberate (BS3; Pierce Chemical Co.) at 22°C for 45 min, and the reaction quenched on ice by addition of Tris-HCl, pH 7.2, to 50 mM, followed by further incubation for 15 min. Proteins were immunoprecipitated with either rabbit anti-GFP (1:200; Seedorf et al., 1999), rabbit anticofactor D (1:20), or preimmune sera. Cross-linked and/or immunoprecipitated proteins were analyzed by Western blotting with one of the following antisera: rabbit anti-HA (1:200; Santa Cruz), rabbit anti-GFP (1:10,000), mouse anti–α-tubulin (1:1,000; Sigma Chemical Co.), or mouse anti–β-tubulin (1:200; Sigma Chemical Co.).

Results

Arls Are Homologues of a Saccharomyces cerevisiae Protein that Affects Microtubule Behavior

Homologues of tubulin folding cofactors A (*RBL2*; Archer et al., 1995), B (*ALF1*; Tian et al., 1997), D (*CIN1*; Hoyt et al., 1990; Stearns et al., 1990) and E (*PAC2*; Hoyt et al., 1997), but not C, have been identified in *S. cerevisiae*, although there are clearly many important differences between mammalian and yeast tubulin folding pathways (Lewis et al., 1997; Cowan and Lewis, 1999). We used the homology search algorithm psi blast, which was specifically created for the detection of weak homologies (Altschul et al., 1997): this identified Cin2p as a possible homologue of cofactor C. Cofactor C and Cin2p share 14% amino acid sequence identity and 32% similarity over 60%

Figure 1. A, Comparison of the sequence of Cin4p with that of four human ARF-like proteins, Arl2-5. B, Interaction of Arl2-5 with cofactor D. Analysis on 12% SDS gels of immune precipitates formed by reaction of ^{35}S-labeled Arls with cofactor D and immunoprecipitated with anticofactor D antibody. T, Control translation reactions; PI, immune precipitates formed by preimmune antisera; I, immune precipitates formed by immune antisera. The faster migrating Arl2 band contained in the doublet generated by in vitro translation probably represents internal initiation of Arl2 at Met[10].

of their length. Genetic experiments in yeast have shown that *CIN1* (D) and *CIN2* (C) act in concert with a third gene, *CIN4*, in a pathway affecting microtubule stability (Hoyt et al., 1990, 1997). Because of our interest in cofactors C (*CIN2*) and D (*CIN1*), we decided to investigate mammalian homologue(s) of *CIN4*. A database search to identify human Cin4p homologues revealed a family of small G proteins, including Arl2, Arl3, Arl4, and Arl5 (Fig. 1 A).

Arl2 Interacts with Cofactor D

We cloned full-length cDNAs encoding human Arl2, Arl3, Arl4, and Arl5, labeled the corresponding proteins by transcription/translation in vitro, and incubated them with added cofactor D. We found that Arl2 (but not Arl3, Arl4, or Arl5) was immunoprecipitable with our anticofactor D antibody (Fig. 1 B), suggesting that Arl2 (Clark et al., 1993) is the true homologue of Cin4p. We found that Arl2 could not complement yeast cells for the loss of *CIN4*. However, its overexpression in a *CIN4* deletion strain resulted in increased supersensitivity to the microtubule poison benomyl, whereas Arl3 had no such effect (Bhamidipati, A., F. Bartolini, and N. Cowan, unpublished observations). These data suggest that Arl2 may be acting in a dominant negative fashion because of its weak homology with *CIN4*.

To further characterize the interaction between Arl2 and cofactor D, we analyzed the products of an Arl2 in vitro translation reaction on a gel filtration column. The majority of labeled Arl2 migrated as a monomer with an apparent mass of 20 kD, with a minor radioactive peak (which could represent Arl2 complexed with one or more cofactors present in the reticulocyte lysate) migrating in the range 160–200 kD (Fig. 2 A). In an Arl2 translation reaction incubated with cofactor D before gel filtration, we found a fourfold enhancement (relative to the control) in the size of the 160–200-kD peak (Fig. 2, A–C). This labeled material was immunoprecipitable with anticofactor D antibody (Fig. 2 D). These data demonstrate the formation of a stable complex containing Arl2 and cofactor D.

To investigate the possible nucleotide dependence of the interaction between Arl2 and cofactor D, we generated the Arl2 mutants Q70L and T30N (numbers refer to the corresponding amino acids in Arl2), corresponding to the classical Ras mutations Q61L and T17N. These mutations have the same effect on many small G proteins: Q61L-type mutations are GTPase defective (GTP remains bound), whereas T17N are defective in GTP binding and, when expressed in vivo, act in a dominant negative manner, sequestering guanine nucleotide exchange factors, so that both mutant and endogenous proteins remain primarily GDP-bound (Bourne et al., 1990; Boguski and McCormick, 1993). We found that His-tagged Arl2-T30N completely failed to take up GTP, consistent with the GTP exchange properties of the same mutation in other small G proteins, while His-tagged Arl2-Q70L exchanged GTP two to three times faster than His-tagged wild-type Arl2. None of these proteins had measurable intrinsic GTPase activity (data not shown). These mutant Arl2 proteins, His-tagged at their COOH terminus, were incubated with cofactor D translated in vitro, and then isolated by binding to an affinity resin. As shown in Fig. 2 E, cofactor D bound to the wild-type and T30N forms of Arl2, but only weakly to the GTPase defective form Q70L. This result suggested that GDP-Arl2 interacts preferentially with cofactor D.

To verify that cofactor D is indeed an effector of the G protein Arl2, we made two mutations (T47A and F50A) in the putative effector loop of Arl2. Residue T47 in Arl2 corresponds to T35 in Ras, and is completely conserved among all members of the Ras superfamily (Pai et al., 1989). This residue plays a critical role in the conformational switch that occurs between the GDP-bound and GTP-bound forms. To confirm our conclusions based on experiments using the Arl2 T30N mutant (namely, that GDP-Arl2 interacts preferentially with cofactor D), we tested the ability of T47A to bind to cofactor D, and found that it interacts in a manner indistinguishable from wild-type Arl2 (Fig. 2 F, left and center). We conclude that the ability of Arl2 to switch from the GDP-bound to the GTP-bound conformation is not essential for binding to cofactor D.

Residue F50 in Arl2 is conserved in all ARF family members and has been implicated in maintaining the integrity of the GDP-bound state, but is absent from many G proteins in the Ras superfamily (Amor et al., 1994; Goldberg, 1998). Therefore, we tested the ability of a mutated Arl2, F50A, to bind to cofactor D, with the expectation that such binding would be abrogated because of disruption of the loop required for maintenance of the proper conformation of Arl2 in its GDP-bound state. This expectation was borne out experimentally (Fig. 2 F, right). We conclude that cofactor D is an effector of Arl2-GDP.

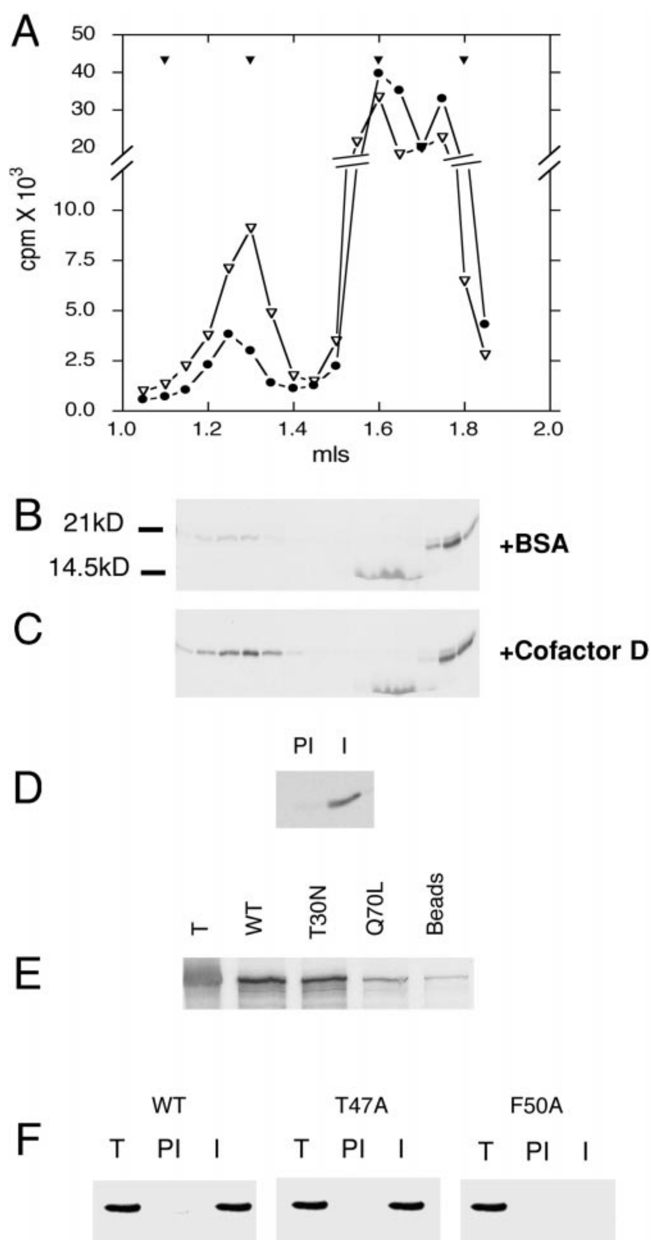

Figure 2. A, Analysis by gel filtration of the products of transcription/translation reactions programed with Arl2 and incubated with BSA (as a control; closed circles) or with cofactor D (open triangles). The position of molecular mass markers (left to right: thyroglobulin, 670 kD; bovine IgG, 158 kD; chicken ovalbumin, 44 kD; equine myoglobin, 17 kD) is shown (closed triangles). B and C, Analysis by 12% SDS-PAGE of the fractions shown in B. The peak comigrating with the ovalbumin marker is hemoglobin, which is an endogenous product of the reticulocyte transcription/translation cocktail. Molecular mass markers are shown at the left. D, Autoradiogram of a 12% SDS polyacrylamide gel of the products of an immune precipitation reaction done with anticofactor D antibody and material contained in the 160–200-kD peak generated in a reaction containing added cofactor D. PI, Preimmune antisera; and I, immune antisera. E, Differential binding of translated cofactor D to Arl2 mutant proteins. His-tagged Arl2 proteins were incubated with radiolabeled translated cofactor D and complexes were isolated on an affinity resin. Bound material was analyzed by SDS-PAGE, followed by autoradiography. F, An Arl2 variant containing a mutation in the putative effector loop fails to bind cofactor D. HA-tagged wild-type Arl2 and the corresponding mutations T47A and F50A were translated in vitro, incubated with cofactor D, and immunoprecipitated with an anticofactor D antibody, and the immunoprecipitated material resolved by SDS-PAGE. T, In vitro translation product; PI, preimmune antisera; and I, immune antisera.

Arl2 Regulates the GAP Activity of Cofactors C, D, and E, and Prevents the Interaction of Cofactor D with Native Tubulin

Cofactors C, D, and E not only participate in the de novo folding of tubulin, but also interact with the native dimer, stimulating GTP hydrolysis by β-tubulin in a polymerization-independent reaction (Tian et al., 1999). Because Arl2 interacts with cofactor D, we examined the effect of purified Arl2 on cofactor-stimulated GTP hydrolysis by tubulin. We found that addition of increasing concentrations of Arl2 to a reaction containing tubulin and cofactors C, D, and E caused an incremental inhibition in the relative rate of GTP hydrolysis. In contrast, in parallel control reactions, Arl3, which does not interact with cofactor D in vitro (Fig. 1 B), had no effect on the tubulin-GAP activity of cofactors at the highest concentration tested (Fig. 3 A). These data give functional significance to the interaction of Arl2 with cofactor D described above.

Because Arl2 interacts with cofactor D and inhibits the tubulin GAP activity, it seemed likely that Arl2 might prevent the interaction between cofactor D and the β-subunit of native tubulin (Tian et al., 1996). We tested this hypothesis by analyzing the products of reactions in which tubulin dimers ^{35}S-labeled in the β-subunit by translation in vitro were allowed to react with cofactor D in the absence or presence of Arl2. We found that the generation of the characteristic cofactor D/β-tubulin complex was indeed inhibited by the addition of increasing amounts of Arl2, with the appearance of a small amount of a new product which presumably consists of β-tubulin, cofactor D, and Arl2. In contrast, the addition of a control protein (GST) to the reaction had no detectable effect (Fig. 3 B). We conclude that Arl2 indeed inhibits the interaction of cofactor D with native tubulin dimers.

Microtubule Destruction in Cultured Cells Expressing Cofactors D and E

To explore the consequences of modulating the expression of cofactors C, D, and E in vivo, we engineered GFP fusion constructs (pGFP-C, pGFP-D, and pGFP-E) and transfected them into HeLa cells. Overexpression of cofactor C had no noticeable effect on the microtubules of transfected cells (data not shown). Remarkably, however, we found that overexpression of either cofactor D or E resulted in the partial or complete loss of tubulin dimer and microtubules (Fig. 4, A–L). Cells in which all microtubules were destroyed as a result of transfection with pGFP-D or pGFP-E showed little or no trace of cytosolic label when stained with an α-tubulin antibody (Fig. 4, F and J). On the other hand, staining of pGFP-D–transfected cells with an anti–β-tubulin antibody showed diffuse cytosolic labeling, whereas pGFP-E–transfected cells had a lower level of diffuse β-tubulin labeling (Fig. 4, H and L). We interpret this diffuse labeling as cofactor D/β-tubulin complexes: we ob-

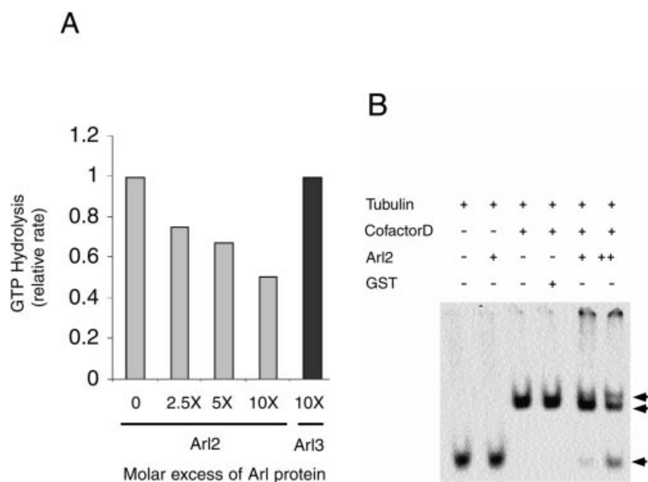

Figure 3. A, Arl2, but not Arl3, suppresses the cofactor-induced GTPase activity of tubulin. Relative rates of GTP hydrolysis in reactions containing tubulin, cofactors C, D, and E, and the molar excess (with respect to cofactor D) of Arl 2 or Arl3 shown. Each relative rate was calculated as an average from two or more independent experiments. B, Arl2 prevents the interaction of cofactor D with native tubulin. Purified native tubulin dimers ^{35}S-labeled in the β-subunit by in vitro translation were incubated with cofactor D in the absence or presence of either GST (as a control) or a 5- or 15-fold molar excess (with respect to cofactor D) of purified Arl2. Reaction products were analyzed on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography. Arrows (top to bottom) show the position of Arl2/β-tubulin/cofactor D complex, β-tubulin/cofactor D complex, and native tubulin dimers, respectively.

served the same destruction of tubulin dimer when untagged cofactor D was overexpressed in HeLa cells, and in this case cofactor D copurified with β-tubulin from these cells as a complex (Tian, G., and N.J. Cowan, unpublished data). These observations are consistent with the fact that cofactors D and E can disrupt the native heterodimer in vitro, sequestering either the α (cofactor E) or β (cofactor D) polypeptides and destabilizing the freed subunit. The cofactor D/β-tubulin complex thus formed can be isolated biochemically as a stable entity, whereas the corresponding cofactor E/α complex is intrinsically unstable (Lewis et al., 1997). Therefore, overexpression of pGFP-E leads to the loss of α-tubulin and the accumulation of a small amount of β-tubulin complexed with endogenous cofactors, whereas overexpression of pGFP-D causes the accumulation of GFP-D/β-tubulin complexes and the obliteration of α subunits.

To show that the levels of tubulin, and not just microtubules, were indeed affected by overexpression of cofactors, we performed parallel experiments in which the transfected cells were treated with nocodazole 1.5 h before fixation. These conditions resulted in complete microtubule depolymerization. We found that cells expressing GFP-D or GFP-E lost virtually all detectable α-tubulin (Fig. 5, B and F). On the other hand, staining of transfected cells with an anti–β-tubulin antibody showed the presence of abundant residual β-tubulin (Fig. 5, D and H). Neither α- or β-tubulin can exist on their own as stable entities (Tian et al., 1997); therefore, in the case of cells transfected with pGFP-D, the β-tubulin must be complexed with overexpressed cofactor D (Fig. 5 D; see below), whereas in cells overexpressing cofactor E (Fig. 5 H), the β-tubulin is presumably complexed with endogenous cofactor D or other cofactors (such as cofactor A; Gao et al., 1994) capable of stabilizing the free β-subunit. The fact that the β-tubulin signal is weaker in pGFP-E transfected cells compared with cells transfected with pGFP-D presumably reflects the relative superabundance of cofactor D in the latter case.

Cofactor D Forms a Stable Complex with β-Tubulin In Vivo

To further test our conclusion that a stable GFP-cofactor D/β-tubulin complex is generated in vivo as a result of overexpression of cofactor D, we prepared extracts of pGFP-D and pGFP-E transfected cells and incubated them with an anti-GFP antibody. Recovered immunoprecipitated material was then analyzed for its content of α- or β-tubulin. We found that anti-GFP–immunoprecipitated material from cells transfected with pGFP-E contained no detectable α-tubulin, consistent with the unstable nature of the cofactor E/α-tubulin complex. In contrast, anti-GFP immunoprecipitated material from pGFP-D–transfected cells contained appreciable quantities of β-tubulin (Fig. 6). These data are completely consistent with our previous work with the corresponding purified untagged cofactor proteins in vitro (Tian et al., 1996, 1997, 1999), and confirm that overexpression of GFP-D in cultured cells results in the accumulation of β-tubulin subunits as stable GFP-D/β-tubulin complexes.

Coexpression with Arl2 Rescues Microtubules from Destruction by Cofactor D

To study the interaction of Arl2 with cofactors in vivo, a plasmid (pHA-Arl2) encoding Arl2 tagged with an HA epitope was cotransfected with either pGFP-D or pGFP-E. In this experiment, expression of HA-Arl2 prevented the loss of microtubules caused by the overexpression of GFP-D (Fig. 7, A–C). In contrast, cotransfection with pHA-Arl2 failed to rescue the microtubule network in cells overexpressing GFP-E, with which it does not interact in vitro (data not shown). Identical results were obtained using constructs engineered for the expression of untagged Arl2. To see if this rescue is specific to Arl2, we cotransfected pGFP-D with a plasmid (pHA-Cdc42) encoding a G protein of the Rho family, Cdc42, also tagged with HA. HA-Cdc42 failed to rescue microtubules from their destruction caused by expression of GFP-D (Fig. 7, D–F). We conclude that Arl2 specifically inhibits the interaction of cofactor D with native tubulin in vivo, as it does in vitro (see above), thereby averting the destruction of the tubulin heterodimer caused by excess cofactor D.

Arl2 Forms a Complex with Cofactor D In Vivo

Because Arl2 interacts with cofactor D in vitro (Fig. 2) and rescues microtubules from destruction by overexpression of cofactor D (Fig. 7), we wanted to demonstrate the existence of an Arl2/cofactor D complex in vivo. To do this, we made extracts from cells cotransfected with pHA-Arl2 and pGFP-D. These extracts were incubated with the cross-

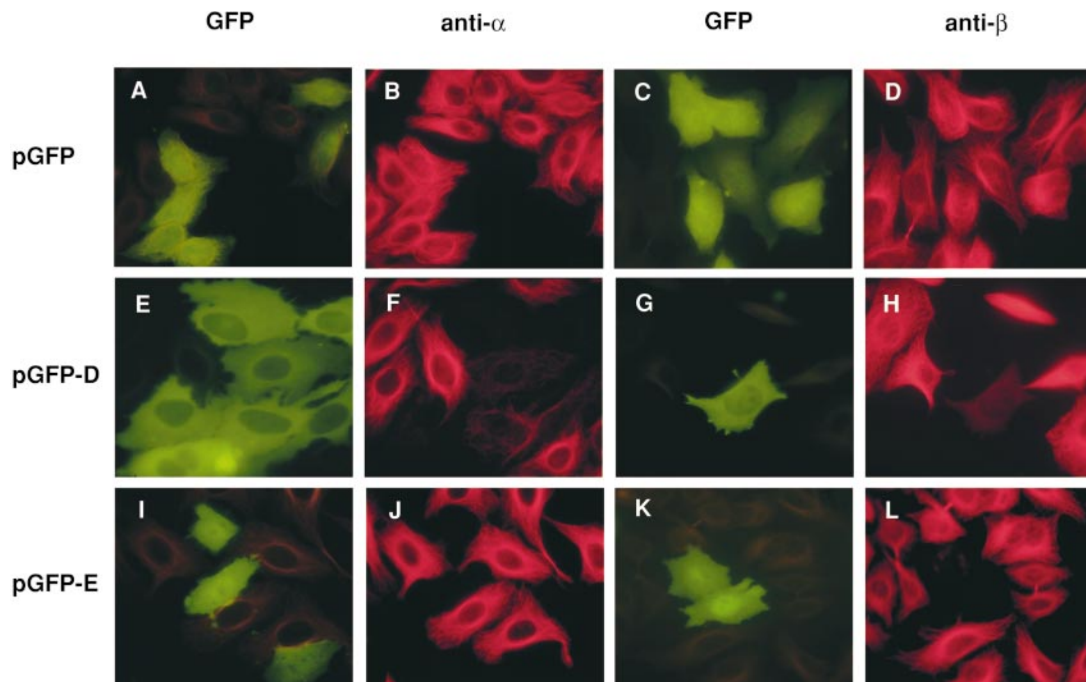

Figure 4. Overexpression of cofactors D or E causes microtubule destruction. Double-label immunofluorescence of HeLa cells transfected with pGFP alone (as a control: A–D), pGFP-D (E–H), or pGFP-E (I–L). Microtubules are shown in red, detected with either an anti–α-tubulin antibody (B, F, and J) or an anti–β-tubulin antibody (D, H, and L).

linking reagent BS3 and the reaction products analyzed by Western blotting with anti-HA or anti-GFP antibodies. Upon cross-linking, a product with a molecular mass corresponding to approximately the sum of the molecular masses of GFP-D and HA-Arl2 appeared in each case (Fig. 8 A). These data imply the existence of an Arl2/cofactor D complex in our cell extracts. To confirm this, we incubated the cross-linked extract with anticofactor D antibody, and assayed the immunoprecipitated material by Western blotting with an anti-HA antibody. This experiment (Fig. 8 B) shows that the cross-linked product contains cofactor D and Arl2. We conclude that Arl2 and cofactor D form a complex in vivo.

Phenotypic Consequences of the Expression of Arl2 and Arl2 Mutants In Vivo

To investigate the possible role of Arl2 in vivo, constructs

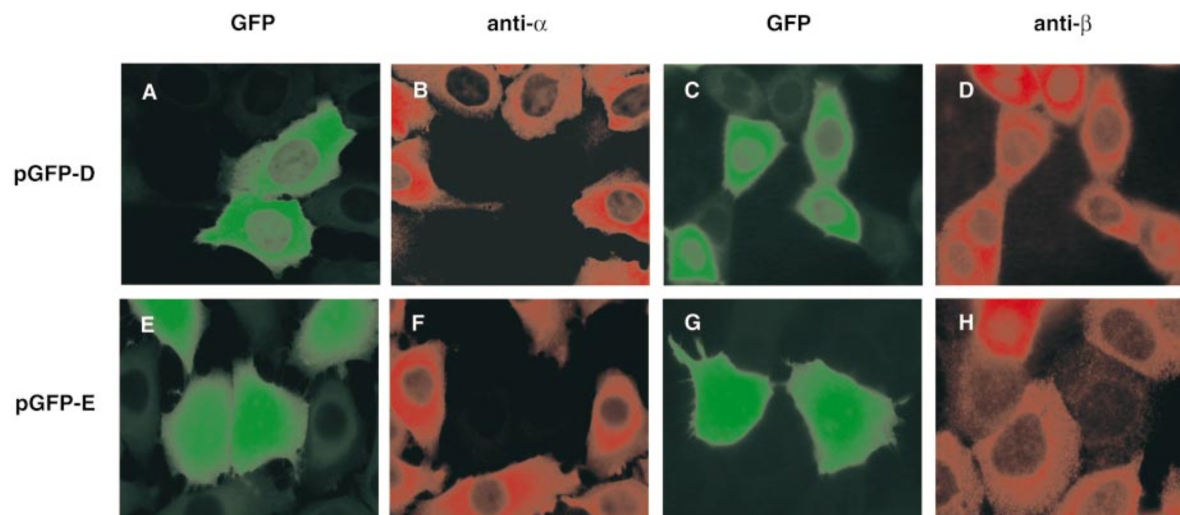

Figure 5. Overexpression of cofactors D or E results in the loss of tubulin dimers. Double-label immunofluorescence of HeLa cells transfected with pGFP-D (A–D) or pGFP-E (E–H) and treated with nocodazole before fixation. α- or β-tubulin was detected with an anti–α- (B and F) or anti–β-tubulin (D and H) mAb. Note the virtual disappearance of detectable α-tubulin signal in cells transfected with pGFP-D or pGFP-E (B and F), whereas there is retention of a strong β-tubulin signal in cells transfected with pGFP-D (D), and a weaker β-tubulin signal in cells transfected with pGFP-E (H).

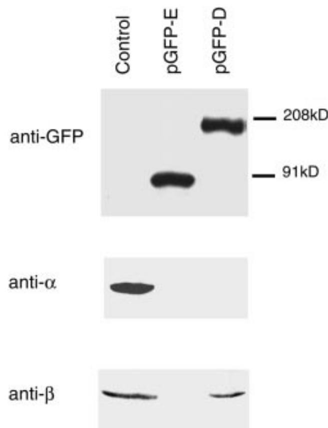

Figure 6. Cofactor D/β-tubulin complexes can be immunoprecipitated from cells transfected with pGFP-D, but no corresponding stable α-tubulin containing complexes can be isolated from cells transfected with pGFP-E. Extracts prepared from cells transfected with pGFP-D or pGFP-E were incubated with an anti-GFP antibody and the immune precipitates analyzed by Western blotting with an anti-GFP antibody (top), an anti–α-tubulin antibody (middle) or an anti–β-tubulin antibody (bottom). An extract from untransfected cells was used on the Western blot as a control. Note the detection of β-tubulin from the pGFP-D transfected cell extract, in contrast to the lack of detectable α-tubulin from the pGFP-E transfected cell extract.

for the expression of GFP-tagged wild-type Arl2 or Arl2 mutants Q70L and T30N (described above) were transfected into HeLa cells. Expression of these proteins in transfected cells had no obvious effect on microtubules (data not shown). Cotransfection of HA-tagged Arl2 constructs with pGFP-D resulted in the same pattern of activity seen in the cofactor D binding experiments (Fig. 2): HA-Arl2-T30N, together with pGFP-D, prevented microtubule destruction caused by expression of cofactor D as effectively as HA-Arl2. In contrast, the GTPase defective Arl2 mutant (HA-Arl2-Q70L) failed to rescue cofactor D-induced microtubule destruction (Table I). Since HA-Arl2-Q70L is GTP-bound and does not rescue,

Table I. Effect of Expression of Arl2 Mutants on Cofactor D-induced Microtubule Destruction In Vivo

Cotransfected gene	Cotransfected cells ± SD showing complete microtubule destruction*
	%
Arl2 (wild-type)	26 ± 10
Arl2 (T30N)	19 ± 8
Arl2 (Q70L)	80 ± 7
Arl2 (T47A)	27 ± 3
Arl2 (F50A)	80 ± 7
Cdc42 control	87 ± 10

*Each result is the average from three or more independent transfection experiments.

whereas HA-Arl2-T30N is presumably primarily GDP-bound and does rescue, we infer that, to prevent the catastrophic activity of cofactor D, Arl2 must be GDP-bound. We also did cotransfection experiments using the HA-tagged Arl2 effector mutations T47A and F50A described (see Fig. 2). Cotransfection of pGFP-D and T47A (which binds cofactor D; Fig. 2 F) results in microtubule rescue, whereas cotransfection of pGFP-D and F50A (which fails to bind cofactor D; Fig. 2 F) does not rescue microtubules (Table I). These data reinforce our conclusion that cofactor D interacts with GDP-Arl2 in vivo.

Discussion

The functions of any member of the large family of mammalian ARF-like G proteins (Arls) have yet to be determined. Here, we have shown that one member of this family, Arl2, interacts with the tubulin-specific chaperone cofactor D, prevents the destruction of tubulin by cofactor D in vivo, and inhibits the tubulin GAP activity of cofactors in vitro. The only previous report on Arl2 effector

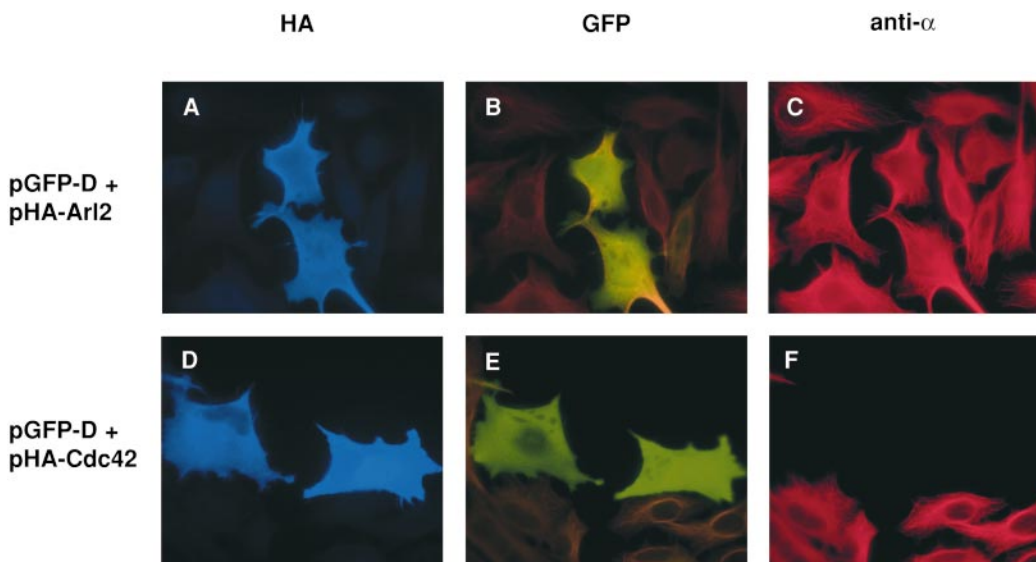

Figure 7. Expression of Arl2 rescues microtubules from destruction by overexpression of cofactor D. Triple label immunofluorescence of HeLa cells transfected with pGFP-D and either pHA-Arl2 (A–C) or pHA-Cdc42 (D–F). HA-Cdc42 and HA-Arl2 (Pai et al., 1989), detected with an anti-HA antibody, are shown in blue; microtubules (detected with an anti–α-tubulin antibody) are shown in red. Note that (in contrast to transfected cells expressing GFP-D alone, see Fig. 3) transfected cells coexpressing GFP-D and Arl2 have a normal microtubule phenotype.

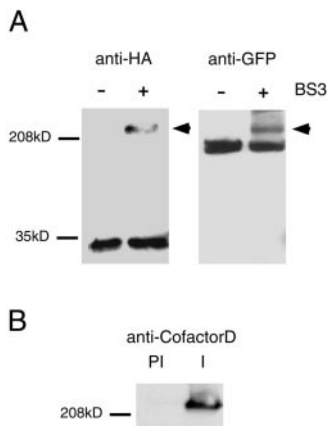

Figure 8. Arl2 forms a complex with cofactor D in vivo. A, Western blot analyses of extracts prepared from cells cotransfected with pGFP-D and pHA-Arl2. Extracts were incubated without (−) or with (+) the cross-linking reagent BS3 before resolution by SDS-PAGE. Detection was with either anti-HA antibody (left), or anti-GFP antibody (right). Arrows highlight the band corresponding to the cross-linked Arl2-cofactor D product. B, The cross-linked product in cells cotransfected with p-GFP-D and HA-Arl2 contains both Arl2 and cofactor D. An extract from cells cotransfected with p-GFP-D and pHA-Arl2 was subjected to cross-linking with BS3 and incubated with a preimmune (PI) or immune (I) anticofactor D antibody. Immunoprecipitated material was analyzed by Western blotting using an anti-HA antibody. Molecular weight markers are shown on the left.

proteins showed that Arl2 binds to BART (binder of Arl two), but the phenotypic consequences of this interaction are unknown (Sharer and Kahn, 1999).

While there are six ARF or Arl related proteins in *S. cerevisiae*, the fact that Arl2 interacts with cofactor D (Figs. 1 and 2), mirroring the genetic interaction of *S. cerevisiae* Cin4p and Cin1p (Hoyt et al., 1997), implies that Arl2 is the homologue of yeast Cin4p. However, there are dramatic differences among *S. cerevisiae*, *Schizosaccharomyces pombe*, and mammals with regard to the roles of tubulin-folding cofactors (Lewis et al., 1997; Cowan and Lewis, 1999). None of the tubulin-folding cofactors are essential for the viability of *S. cerevisiae* (Hoyt et al., 1990, 1997; Stearns et al., 1990; Archer et al., 1995; Tian et al., 1997; Feierbach et al., 1999), although the three tested so far (cofactors B, D, and E) are essential in *S. pombe* (Hirata et al., 1998; Radcliffe et al., 1999), and all five cofactors are likely to be so in most eukaryotes, given the high conservation of tubulin and the fact that in vitro, tubulin cannot be folded to the native state in their absence (Tian et al., 1997). There are other differences as well: whereas we find that overexpression of cofactor D destroys tubulin and microtubules (Fig. 4), in *S. cerevisiae* overexpression of its homologue results in mild microtubule instability (Hoyt et al., 1990, 1997; Stearns et al., 1990). Overexpression of the cofactor D homologue Alp1 in *S. pombe* is lethal (Hirata et al., 1998), but results in abnormal microtubule structures; in this organism, Alp1 binds to microtubules, whereas cofactor D does not bind to microtubules in mammalian cells. Overexpression of cofactor E homologues has no effect in either yeast species (Grishchuk and McIntosh, 1999; Radcliffe et al., 1999), but in mammalian cells, tubulin and microtubules are obliterated (Fig. 4). Because of these differences in the actions of cofactors in yeasts and in mammals, understanding the roles of cofactors and Arl2 in mammalian cells is particularly important.

A model incorporating the action of Arl2 on the tubulin folding and polymerization pathways is presented in Fig. 9. Tubulin subunits are folded to a quasinative state by the chaperonin CCT, assisted by the chaperone protein prefoldin. The tubulin-specific chaperones (cofactors A–E) then assemble the native tubulin heterodimer. The release of tubulin from chaperones occurs upon hydrolysis of GTP by the bound tubulin (Lewis et al., 1997; Tian et al., 1997). In addition to functioning in tubulin folding pathways, cofactors can interact with native tubulin in two ways: cofactor D or E in excess will destroy the tubulin dimer by sequestering the β or α subunit, respectively, leading in each case to the destabilization of the freed subunit (Tian et al., 1997); or cofactors C, D, and E together act as a GTPase activating protein (GAP) for tubulin (Tian et al., 1999), converting GTP tubulin, which is capable of polymerization, into GDP tubulin, which is not.

This much of our model was deduced from biochemical experiments using purified components (Cowan and Lewis, 1999). The in vivo data presented here extends the model: the obliteration of tubulin caused by overexpression of cofactor D or E in transfected cells results from the interaction of cofactors with native tubulin, as it does in vitro. Here, we also show that coexpression with Arl2 prevents tubulin destruction by cofactor D in vivo (Fig. 4), implying that Arl2 regulates the interaction of cofactor D with native tubulin. This conclusion is reinforced by the fact that in vitro, Arl2 inhibits the tubulin-GAP activity of cofactors C, D, and E, and inhibits the interaction of cofactor D with tubulin dimer (Fig. 3). Thus, the negative regulation by Arl2 is indicated in Fig. 9 in two places. In contrast, Arl2 has no effect on tubulin folding in vitro, suggesting that the tubulin-GAP activity can be regulated even as de novo folding proceeds.

The experiments using GTPase defective and GTP-binding defective mutants of Arl2 show that it is the GDP-bound form of Arl2 that preferentially interacts with cofactor D. The GTPase defective mutant Q70L, which is GTP-bound, binds poorly to cofactor D in vitro, whereas the mutant T30N, which is defective in GTP binding, binds cofactor D in a manner indistinguishable from wild-type Arl2 (Fig. 2 E). Furthermore, when a threonine residue is altered in the putative effector loop of Arl2 that is needed for the conformational change that accompanies GTP binding, the mutant protein can still bind cofactor D as efficiently as wild-type Arl2. This threonine residue falls within a domain placed such that its hydroxyl group interacts with the Mg^{2+} ion and the β- and γ-phosphates of the bound GTP (Pai et al., 1989; Goldberg, 1998). Mutations at this position in Ras-like proteins abolish binding to many of those effectors that bind exclusively to GTP-bound G proteins. Thus, the binding of the T47A mutant to cofactor D is consistent with the results obtained with the T30N and Q70L mutants: all point to the interaction of cofactor D with the GDP-bound form of Arl2. Furthermore, mutation of a phenylalanine residue (F50) that resides in the same effector loop results in a complete failure to bind cofactor D (Fig. 2 F). Residue F50 in Arl2 corresponds to residue F51 in ARF1, and is part of a beta strand and beta turn in ARF1 (Amor et al., 1994; Goldberg, 1998) whose sequence is absolutely conserved in the ARF family of GTPases, but less so in the Arl proteins. This domain is absent from many members of the Ras superfam-

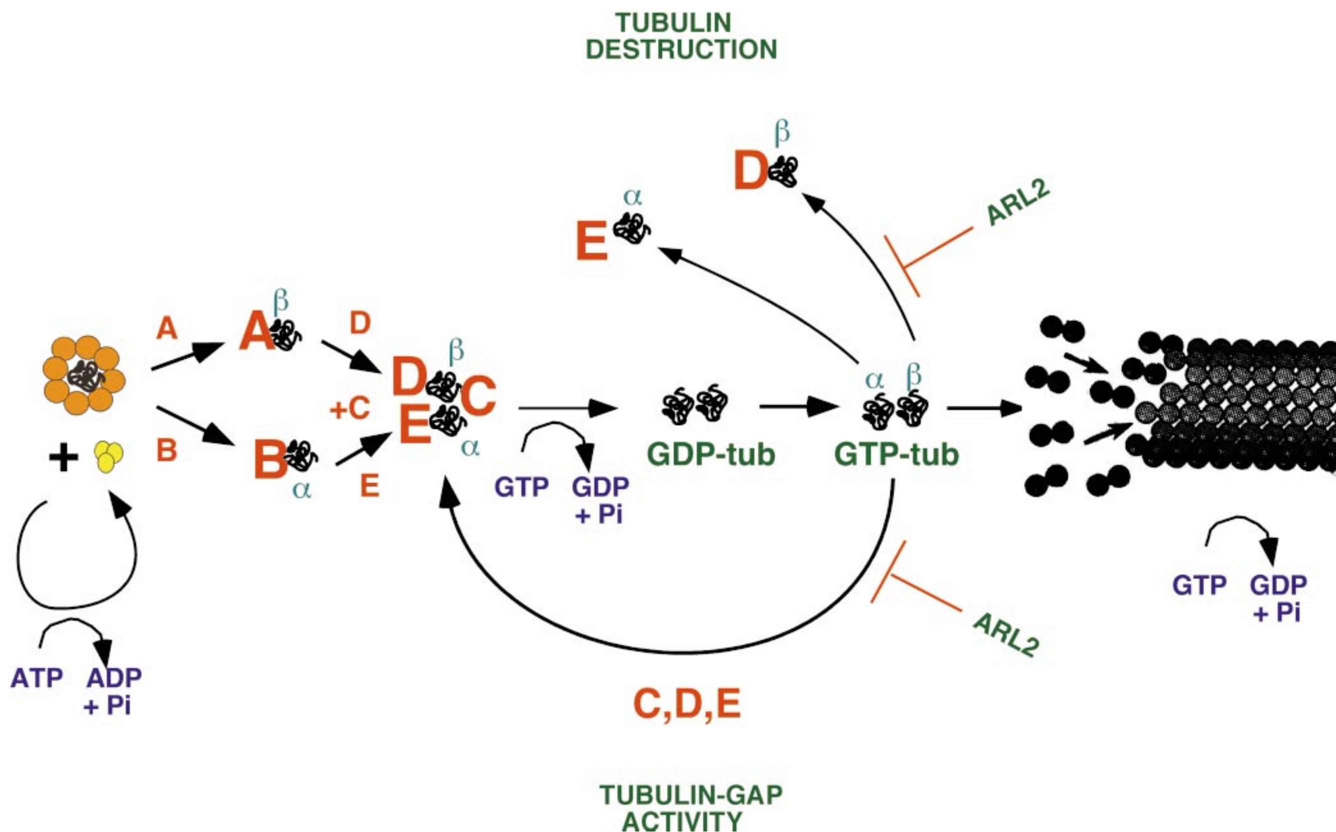

Figure 9. Model depicting the action of Arl2 in the reactions involved in the assembly of the tubulin heterodimer and modulation of its guanine nucleotide state. The chaperonin CCT is shown in orange, prefoldin/GimC is in yellow, and cofactors are denoted by red letters.

ily. As a result, ARF proteins have a unique geometry in their GDP-bound states (Amor et al., 1994). Thus, the failure of cofactor D to bind Arl2-F50A reinforces our conclusion that cofactor D is an effector of GDP-Arl2. These observations were borne out by our in vivo experiments, where we found that only those mutant forms of Arl2 that bound to cofactor D in vitro could rescue microtubules from the catastrophic effects of overexpression of cofactor D (Table I). The rescue function of Arl2 must be mediated via a direct interaction with cofactor D, since Arl2 fails to rescue tubulin from similar destruction by cofactor E, with which it does not interact directly.

Conversion of GTP-tubulin to GDP-tubulin via its interaction with cofactors could be used by the cell in the spatial or temporal control of its microtubule network, since only GTP-tubulin is capable of polymerizing into microtubules, and microtubule stability depends in part on the pool of available GTP-tubulin. Since tubulin readily exchanges its bound nucleotide, the effect of the GAP activity of cofactors (Fig. 9) would be enhanced by the action of a guanine nucleotide exchange inhibitor. The data presented here show that Arl2 inhibits the conversion of GTP-tubulin to GDP-tubulin by cofactors. The fact that the tubulin-GAP activity of cofactors is regulated implies that this reaction indeed contributes to modulating microtubule dynamics.

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