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) proteins are defined by their ability to act as cofactors in the cholera toxin-catalyzed ADP-ribosylation of Gs, 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 A TP 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...
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 (GA Ps; 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 A rl2 or an A rl2 mutant defective in GTP binding (but not a GTPase defective A rl2 mutant) specifically prevents the destruction of tubulin and microtubules caused by expression of cofactor D. In addition, an A rl2 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, A rl2 downregulates the GAP activity of cofactors C, D, and E in vitro. These data establish a role for A rl2 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 cDNA’s encoding cofactors C, D, or E (Tian et al., 1996) into the plasmid pEGFP-C3 (CLONTECH Laboratories, Inc.). Human A rls were cloned (by PCR) into pET2b (Novagen) using human tests mRNA (CLONTECH Laboratories, Inc.) as template; mutant forms of A rl2 were generated by PCR and checked by DNA sequencing. For transfection assays, wild-type and mutant forms of A rl2 were cloned into the plasmid pcDNA3 (CLONTECH Laboratories, Inc.) containing an NH2-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 pET2b (Novagen).

Protein Expression and Purification

Tubulin and cofactors C, D, and E were purified as described previously (Tian et al., 1996). A rl2 and A rln were purified from extracts of host E. coli BL21D3 (A rl2) or BL21DE3LYE (A rln) cells cleared by centrifugation at 100,000 g at 4°C for 30 min with purified cofactor D. 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).

GTPase Assays

Rates of GTP hydrolysis were measured in reactions done as described (Tian et al., 1999), using γ-32P-labeled GTP (specific activity, 6.0 mCi/μmol) and purified bovine brain tubulin (17 μM) with or without added cofactors (C, 0.40 μM; D, 0.13 μM; E, 0.26 μM) and A rl2 or 3 (0.5, 1.0, 2.0 μM).

Reaction of Cofactor D with Native Tubulin In Vitro

Purified tubulin heterodimer, 32P-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 10-fold molar excess (with respect to cofactor D) of purified recombinant A rl2. 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). A 20 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); 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 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 antiafactor D (1:200), 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; Sigma Chemical Co.), mouse anti–α-tubulin (1:200; 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 (Itschul 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%
Arl2 Regulates Tubulin Binding by 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 translation reaction in vitro, and incubated with cofactor D before gel filtration. We found a fourfold enhancement (relative to the control) in the range 160–200 kD (Fig. 2 A). In an Arl2 translation reaction incubated with cofactor D, 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 R as 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 to the G D P-bound to the G T P-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.
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 35S-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-
GFP-D or GFP-E lost virtually all detectable tubule depolymerization. We found that cells expressing transfected cells were treated with nocodazole 1.5 h before tors, we performed parallel experiments in which the bulles, were indeed affected by overexpression of cofac-
entities (Tian et al., 1997); therefore, in the case of cells

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

To further test our conclusion that a stable GFP-cofactor 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 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.

Cocexpression 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 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-
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. 8A). 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. 8B) shows that the cross-linked product contains cofactor D and A rl2. We conclude that A rl2 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 A rl2 in vivo, constructs...
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 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). A β 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.

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; see Fig. 2 F) results in microtubule rescue, whereas cotransfection of pGFP-D and F50A (which fails to bind cofactor D; see Fig. 2 F) does not rescue microtubules (Table I). These data reinforce our conclusion that cofactor D interacts with GDP-Arl2 in vivo.

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

<table>
<thead>
<tr>
<th>Cotransfected gene</th>
<th>Cotransfected cells ± SD showing complete microtubule destruction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arl2 (wild-type)</td>
<td>26 ± 10</td>
</tr>
<tr>
<td>Arl2 (T30N)</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>Arl2 (Q70L)</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>Arl2 (T47A)</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Arl2 (F50A)</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>Cdc42 control</td>
<td>87 ± 10</td>
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</table>

*Each result is the average from three or more independent transfection experiments.
The role 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 B). 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 A R F 1, and is part of a beta strand and beta turn in A R F 1 (Amor et al., 1994; Goldberg, 1998) whose sequence is absolutely conserved in the A R F family of GTPases, but less so in the A r l proteins. This domain is absent from many members of the R as superfam-
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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