A Trimer Consisting of the Tubulin-specific Chaperone D (TBCD), Regulatory GTPase ARL2, and β-Tubulin Is Required for Maintaining the Microtubule Network*

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Microtubule dynamics involves the polymerization and depolymerization of tubulin dimers and is an essential and highly regulated process required for cell viability, architecture, and division. The regulation of the microtubule network also depends on the maintenance of a pool of αβ-tubulin heterodimers. These dimers are the end result of complex folding and assembly events, requiring the TCP1 Ring Complex (Tric or CCT) chaperonin and five tubulin-specific chaperones, tubulin binding cofactors A–E (TBCA–TBCE). However, models of the actions of these chaperones are incomplete or inconsistent. We previously purified TBCD from bovine tissues and showed that it tightly binds the small GTPase ARL2 but appears to be inactive. Here, in an effort to identify the functional form of TBCD and using non-denaturing gels and immunoblotting, we analyzed lysates from a number of mouse tissues and cell lines to identify the quaternary state(s) of TBCD and ARL2. We found that both proteins co-migrated in native gels in a complex of 200 kDa that also contained β-tubulin. Using human embryonic kidney cells enabled the purification of the TBCD-ARL2-β-tubulin trimer found in cell and tissue lysates as well as two other novel TBCD complexes. Characterization of ARL2 point mutants that disrupt binding to TBCD suggested that the ARL2-TBCD interaction is critical for proper maintenance of microtubule densities in cells. We conclude that the TBCD-ARL2-β-tubulin trimer represents a functional complex whose activity is fundamental to microtubule dynamics.

Microtubules are highly dynamic polymers that are best known for their roles as the central cytoskeletal structure in cells and in mitotic spindles during cell division. They are also the tracks on which organelles traffic, particularly important in polarized cells that generate great distances between parts of the cell. Additionally, they are the core of sensory and motile cilia or flagella. The formation and destruction of microtubules and microtubule bundles are orchestrated by a large number of proteins that include the microtubule-associated proteins. Microtubules are polymers of the αβ-tubulin dimer, with several genes encoding each α- or β-tubulin subunit (e.g. see Lewis et al. (1)), resulting in some diversity in composition. Tubulins can also be modified by posttranslational modifications, including acetylation, tyrosination, and phosphorylation, which can alter the dynamics of the polymerization and depolymerization reactions. Because of the essential role of microtubules in cell division, they have also been the target of many antitumor therapies, e.g. the taxanes and Vinca alkaloids (2). However, despite their importance to cells and in the clinic and decades of research, we still lack a complete molecular-level understanding of the biosynthesis and regulation of the formation of αβ-tubulin.

Tubulins are typically the most abundant proteins in mammalian cells, but the generation of the αβ-tubulin dimer requires a complex series of biosynthetic steps to support proper folding and dimer assembly. Newly synthesized α- and β-tubulin, as well as other proteins (3, 4), first interact with the TCP1 Ring Complex upon exiting the ribosome. The tubulins then interact with the five tubulin-specific co-chaperones, termed cofactors A–E, in a series of interactions first described by Tian et al. (5, 6), using native gels to monitor the formation of different inferred complexes of in vitro translated tubulin. Because of the complexities involved in αβ-tubulin dimer assembly, there is currently no recombinant system capable of generating pure populations of milligram amounts of unmodified tubulin dimers. The development of a molecular model for tubulin folding would reveal the roles played by each of the required components, allow the generation of key biochemical reagents for multiple studies, and is predicted to generate a number of opportunities for therapeutic agent development. Such a model requires the ability to generate each component in a functional state that would then allow reconstitution of the formation of the αβ-tubulin dimer from parts. Perhaps the largest roadblock to this goal is that one of the required chaperones, tubulin-specific chaperone D (TBCD), is insoluble when expressed in Escherichia coli, making purification difficult. We previously showed that human embryonic kidney cells enabled the purification of TBCD (3), but it remained to be determined whether the human TBCD is inherently insoluble or whether this was due to expression in E. coli. Here, we have taken advantage of an orthogonal expression system and human microtubule-negative cell lines to purify human TBCD and evaluate its activity in vitro.

Microtubule assembly proceeds through a series of complex folding and assembly events that first involve the interactions of newly translated tubulin with cofactors A–E (TBCA–TBCE). However, models of the actions of these chaperones are incomplete or inconsistent. We previously purified TBCD from bovine tissues and showed that it tightly binds the small GTPase ARL2 but appears to be inactive. Here, in an effort to identify the functional form of TBCD and using non-denaturing gels and immunoblotting, we analyzed lysates from a number of mouse tissues and cell lines to identify the quaternary state(s) of TBCD and ARL2. We found that both proteins co-migrated in native gels in a complex of 200 kDa that also contained β-tubulin. Using human embryonic kidney cells enabled the purification of the TBCD-ARL2-β-tubulin trimer found in cell and tissue lysates as well as two other novel TBCD complexes. Characterization of ARL2 point mutants that disrupt binding to TBCD suggested that the ARL2-TBCD interaction is critical for proper maintenance of microtubule densities in cells. We conclude that the TBCD-ARL2-β-tubulin trimer represents a functional complex whose activity is fundamental to microtubule dynamics.

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**References**

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2. The abbreviations used are: TBCD, tubulin-specific chaperone D; GTPγS, guanosine 5’-3-O-(thio)triphosphate; BN-PAGE, blue native PAGE; TEV, tobacco etch virus; PD, pulldown.
pressed in bacteria and poorly expressed or unstable in other expression systems and, thus, is not readily available. This is in marked contrast to the other four cofactors, A–C and E (7).

The earliest evidence that TBCD is important to microtubule biology came from genetic screens in model genetic systems. In *Saccharomyces cerevisiae*, screens for altered sensitivity to drugs acting on microtubules identified mutations in the yeast TBCD ortholog CIN1 (8, 9). Mutations in TBCD orthologs were later identified in similar screens performed in *Schizosaccharomyces pombe* (*Alp1*) (10, 11) and *Arabidopsis thaliana* (*TTN1*). Along with these compelling genetic data, the identification of TBCD as one of the five tubulin-specific cofactors required for *in vitro* folding of tubulin (5) confirmed a central role of TBCD in tubulin biosynthesis. More recently, however, TBCD has been implicated in additional cellular roles, including actions at centrosomes (12–14) and at the cell surface (15, 16). Several recent studies have identified a number of point mutations in TBCD found in patients with links to early-onset encephalopathy (17–19) and intractable seizures (20). Throughout the time when roles for TBCD in microtubule biology were being identified, there have also been strong functional links to the ARL2 GTPase, a member of the ADP-ribosylation factor (ARF) family of regulatory GTPases. The same genetic screens that identified mutations in CIN1/Alp1/TTN1 (TBCD orthologs) that altered microtubules also identified mutations in CIN4/Alp41/TTN5, the orthologs of ARL2 in *S. cerevisiae*, *S. pombe*, and *A. thaliana*, respectively (8, 9). Genetic screening similarly identified effects of mutations in ARL2 orthologs in *Caenorhabditis elegans* (21), *Trypanosoma brucei* (22), and *Drosophila melanogaster* (23). Thus, there is strong evidence that ARL2 orthologs play essential roles in tubulin and microtubule biology. However, ARL2 has other essential regulatory roles in cells that both complicate analyses and increase its overall importance to cell biology (24).

Although other members of the ADP-ribosylation factor family are predominantly present in the cytosol as monomers, fractionating as 20-kDa species, we found that the vast majority of ARL2 fractionates with an apparent molecular mass of ≥200 kDa (25). This is in marked contrast to human ARL2 purified from bacteria or HEK cells overexpressing human ARL2, which are stable monomeric proteins (26). Upon purification from bovine tissues, we found that ARL2 remained tightly bound as a heterodimer with TBCD. However, that heterodimer was inactive in the tubulin folding assay and displayed highly unusual guanine nucleotide binding properties in failing to bind the activating ligands GTP or GTPγS (25). Because the ARL2-TBCD heterodimer purified from bovine tissues appeared to be inactive in key respects, required more than 1 week to purify, and yielded only small (microgram) amounts of protein, we sought other means of assessing the quaternary state of TBCD and ARL2 in cells and tissues.

The model for tubulin folding, first proposed by Cowan and co-workers (6), includes the TBCD-TBCE-α-tubulin-β-tubulin tetramer as the penultimate complex to which TBCC binds, resulting in release of the folded αβ-tubulin heterodimer. Prior to this, the unfolded α- and β-tubulin monomers are thought to be maintained in their unfolded states by binding to cofactors B and A, respectively. A recent publication that used recombiant expression of yeast orthologs argues instead that the basal complex for tubulin folding consists of a trimer of TBCD-TBCE-ARL2 (27). To test these models and further the ultimate goal of reconstitution of tubulin folding as a formal demonstration of the roles of each component, we began by assessing the quaternary state of TBCD and ARL2 in freshly prepared cell or tissue lysates. We found evidence of a very stable and novel complex consisting of TBCD, ARL2, and β-tubulin as well as two other multisubunit protein complexes that are described here for the first time.

**Results**

TBCD and ARL2 Are Present in Animal Cells and Tissues in a Complex with an Apparent Molecular Mass of ~200 kDa—To identify the predominant form of ARL2 that exists in mammalian cells, we re-examined the nature of ARL2 complex(es) found in mammalian cells and tissues. We began by analyzing the elution profile of ARL2 by immunoblotting fractions from a size exclusion column (Superdex 200) used to resolve proteins from a clarified lysate of HeLa cells (Fig. 1, *top panel*). The overwhelming majority (estimated at ~90%) of ARL2 eluted as a single peak in the fractions corresponding to an apparent molecular mass of ~200 kDa (peaking in fraction 15 in Fig. 1, *top panel*). Consistent with our previous work (25), little or no ARL2 was evident in fractions corresponding to a 20-kDa monomer (fractions 23 and 24 in Fig. 1, *top panel*). A faint peak of ARL2 signal was seen at slightly higher apparent molecular masses, corresponding to an estimated size of ~40 kDa (fractions 21 and 22 in Fig. 1, *top panel*). In contrast, when human ARL2 is expressed in bacteria, it purifies as a soluble, stable monomer with no evidence of any species greater than 20 kDa (26). Although the larger, ~200-kDa species was observed previously and proved to be abundant in animal cells/tissues (see below), the intermediate, ~40-kDa species was not observed in our earlier study of Sf295 cell lysates (25) or in several other instances and so was not pursued further at this time.

HeLa cells were then transfected with a plasmid directing the overexpression of human ARL2 prior to repeating the gel filtra-
tion analysis (Fig. 1, center panel). No evident increase in the abundance of the ~200-kDa species was seen. However, there was a clear increase in the ~40-kDa species that includes ARL2 and a vast increase in the amount of ARL2 in fractions 23 and 24, corresponding to a monomer. This suggests that ARL2 expressed in mammalian cells can migrate as a monomer and that the larger species represent homo- or hetero-oligomers. Because ARL2 is known to bind TBCD, we then overexpressed both human ARL2 and TBCD in HeLa cells and repeated the analysis. This time, there was a considerable increase in the amount of ARL2 migrating in the range of the ~200-kDa species, consistent with the presence of both ARL2 and TBCD in these fractions (Fig. 1, bottom panel). These results prompted us to examine the nature of the ARL2-TBCD complex in mammalian cells and tissues.

Despite the fact that we have previously purified from mammalian tissue, over the course of several days, a stable complex that includes ARL2 and TBCD, we were concerned about the possibility of subunit dissociation during analysis altering our conclusions regarding the composition of different ARL2 complexes. Therefore, we sought a method that uses distinct characteristics for separation and is more rapid in resolving TBCD and ARL2 complexes under native conditions. We used blue native (BN) PAGE to analyze freshly prepared cell lysates, as it more rapid, yields higher resolution of proteins and complexes over a broad range of apparent molecular weights, allows simultaneous and parallel analyses of multiple samples, and has been used previously in analyses of tubulin and related proteins (28).

Cell and tissue lysates were prepared as described under “Experimental Procedures,” and the resulting soluble fractions (S100) were analyzed by BN-PAGE and immunoblotting for ARL2 and TBCD. Fig. 2A summarizes the results obtained from HEK, mouse Neuro 2A (N2A) neuroblastoma, mouse embryonic fibroblast (NIH-3T3), and human neuroblastoma (SH-SY5Y) cells. Although multiple bands were observed in most cases, the most abundant species of ARL2 and TBCD in each sample shown, with the exception of SH-SY5Y, is a band migrating just below the 242-kDa size marker, and thus quite close to the predicted ~200-kDa species observed after gel filtration. A few other cell lines were also analyzed in this way and yielded more complex patterns, with bands of different apparent sizes being more prominent than that at 200 kDa (data not shown). Thus, although the complex migrating just below the 242-kDa marker is the predominant and even sole immunoreactive band in some cell lines, this is not true in all cell lines tested. A graph of the migration distance versus the log of the molecular mass, with comparison to molecular weight markers, yielded an apparent molecular weight of 200 kDa, so we will use this size in our discussions of this complex hereafter.

We also examined the quaternary structure of TBCD in several animal tissues. Immunoblots of human, bovine, and murine brain as well as murine liver and kidney tissue lysates were probed with our rabbit polyclonal antibody to TBCD (Fig. 2B). In each of these murine tissues, the predominant species is again migrating at ~200 kDa. In contrast, the human and bovine brain lysates contain predominantly a single band of TBCD immunoreactivity migrating at ~400 kDa, very similar in electrophoretic mobility to the predominant species seen in human SH-SY5Y cells (Fig. 2A). Immunoblotting of these samples was also performed from BN-PAGE using our ARL2 and commercial β-tubulin antibodies, but the results were harder to interpret as a result of greater variability and diversity of β-tubulin-containing complexes, respectively, as well as higher backgrounds in both. This issue is largely the result of immunoblotting from native gels, as each antibody yielded much simpler and cleaner results when blotting from SDS-PAGE gels. The stark differences in apparent molecular weight of the TBCD complex seen in human and bovine compared with murine brain lysates were surprising and without explanations found in the literature.

Although not pursued further in this study, the band at ~400 kDa will likely prove to be related, as we find that it immunoblots positive for TBCE and ARL2 (data not shown) in addition to TBCD and, thus, may be related to the complex studied by Al-Bassam and co-workers (27). We note that the 4−16% gradient BN-PAGE gels did a poor job of quantifying species below ~60 kDa, as proteins smear, resulting in decreased sensitivity of immunoblots. These data led us to conclude that TBCD and

FIGURE 2. Immunoblots of cell and tissue lysates resolved in native gels reveal that endogenous TBCD and ARL2 complexes at ~200 kDa are commonly observed and can be the predominant species of each protein detected in this assay. A, detergent extracts of HEK, N2A, NIH-3T3, and SH-SY5Y cells were clarified by centrifugation at 100,000 × g, resolved (30 μg each) in a 4–16% BN-PAGE gel, and immunoblotted with antibodies specific to ARL2 or TBCD, as indicated and described under “Experimental Procedures.” B, murine kidney or liver and murine, bovine, and human brain samples were homogenized and then clarified by centrifugation at 100,000 × g for 1 h at 4 °C. Lysates (30 μg) were resolved using BN-PAGE and immunoblotted with TBCD antibodies. Hs, Homo sapiens; Bt, Bos taurus; Mm, Mus musculus.
ARL2 are present in a species migrating in native gels with an apparent molecular weight of 200 kDa that is present in almost all samples examined and the predominant, if not sole, species seen in some. This prompted a much more detailed analysis of this protein complex, including its purification from recombinant sources.

Recombinant GST-TBCD Co-purifies with αβ- Tubulin—We and others have found previously that TBCD is insoluble when expressed in bacteria. Tian and Cowan (7) have used insect cell expression for bovine TBCD but found low yields and instability. They later used adenoviruses to express bovine or human TBCD in HeLa cells and found β-tubulin to co-purify only with the bovine protein (29). We used adherent HEK cells as a mammalian expression system and transfected them with plasmids directing expression of N-terminal, GST-tagged human TBCD (GST-TBCD). Following expression, GST-TBCD was affinity-purified using glutathione-conjugated Sepharose beads and eluted with glutathione. The GST tag could optionally be removed using the engineered TEV protease cleavage site between the GST tag and the TBCD open reading frame, resulting in a residual 12-amino acid extension at the N terminus of TBCD.

GST-TBCD was initially analyzed by SDS-PAGE with Coomassie Blue staining after affinity purification from HEK cells expressing GST-TBCD alone (Fig. 3A, lane 2) or co-expression with human ARL2 (Fig. 3A, lane 3). Expression of GST alone (data not shown) or co-expression with ARL2 (Fig. 3A, lane 1) resulted in purification of the 28-kDa GST as well as a band at 24 kDa (Fig. 3A, lanes 1–3, asterisk); note the absence of endogenous ARL2 in lanes 1 and 2. The 24-kDa contaminant is predicted to be an endogenous GST present in insect and HEK cells (30) and is observed in all of our affinity-purified preparations using this source. The contaminant is readily removed by gel filtration or ultrafiltration using a filter with a molecular mass cutoff of 30,000 Da, and thus we are confident that this protein is not a component of any of our complexes.

SDS-PAGE analysis of affinity-purified GST-TBCD from HEK cells resulted in the identification of two bands at ~160 and ~55 kDa (Fig. 3A, lane 2). The larger band is GST-TBCD (28 kDa + 133 kDa), based upon prediction and confirmed by immunoblotting (data not shown). The established role of TBCD in tubulin folding and the size of the band at 55 kDa prompted us to immunoblot for α- and β-tubulin. We found that the 55-kDa band was positive for both α- and β-tubulins and thus conclude that the tubulin heterodimer specifically co-purifies with GST-TBCD (Fig. 3B, left lane). This is the first time that TBCD has been shown to bind directly to αβ-tubulin. Note that ARL2 was not evident in the purified preparation of GST-TBCD-α-tubulin-β-tubulin. Although ARL2 is present endogenously in HEK cells, and presumably almost all bound to endogenous TBCD (Fig. 1, top panel), we suspect that there is simply not enough free ARL2 to bind the higher levels of GST-TBCD achieved in this expression system.

The TBCD-α- tubulin-β-tubulin trimer was then analyzed by both BN-PAGE and gel filtration chromatography. GST-TBCD was expressed and purified from HEK cells, and the GST tag was removed with TEV, as it promotes dimerization that could confound interpretations. When analyzed by BN-PAGE and Coomassie Blue staining, the preparation failed to reveal any distinct band(s) despite loading as much as 4 μg of purified protein. Rather, we either saw a faint haze of staining in the higher molecular weight range of the gel or, more consistently, staining that barely entered the gel (Fig. 4A, left lane). Immunoblotting of BN-PAGE gels confirmed the presence of TBCD in the haze, consistent with oligomerization or aggregation (data not shown). When the same preparation was analyzed by its elution profile from a Superdex S200 column, the protein was all found in the void volume, which is again consistent with aggregation or oligomerization. Thus, when TBCD was expressed and purified from HEK cells without excess ARL2, it co-purified with α- and β-tubulin in a complex that appears to be aggregated or oligomerized. Although not shown here, when comparing the levels of αβ-tubulin co-purifying with TBCD (Fig. 3B, left lane) to a purified tubulin standard, it appears that the tubulins co-purify to similar levels. However, we note that it is possible that GST-TBCD co-purifies with a combination of β-tubulin and αβ-tubulin at varying stoichiometries. Nevertheless, the finding that TBCD binds to αβ-tubulin dimers is thought to be an indication of either a residual interaction from the final step in folding/biosynthesis of the tubulin dimer or of the ability of TBCD to bind to the tubulin dimer either when free in solution or perhaps even in a microtubule. This last possibility may explain the ability of TBCD overexpression to cause the loss of cellular microtubules in cultured cells (12, 29, 31, 32).

Co-expression of GST-TBCD and ARL2 Results in the Formation and Ability to Purify a TBCD-ARL2-β-Tubulin Trimer—When GST-TBCD was affinity-purified from HEK cells co-expressing ARL2, we observed three bands in SDS-PAGE gels corresponding to GST-TBCD at ~160 kDa, ARL2 at ~20 kDa, and again a band at ~55 kDa that appeared to co-migrate with that seen in the GST-TBCD preparation lacking ARL2 (Fig.
Purification of TBCD-ARL2-β-Tubulin

3A). Immunoblot analyses confirmed the bands as TBCD, ARL2, and β-tubulin. However, in marked contrast to the previous trimer, the 55-kDa band was strongly immunoreactive with β-tubulin antibody but clearly negative for α-tubulin (Fig. 3B, right lane). We then asked whether there was specificity for any isoform of β-tubulin by subjecting the band at 55 kDa to mass spectrometry after trypic digestion. In two samples, we obtained over 600 spectral counts for β-tubulin peptides. Among these were multiple isoform-specific peptides derived from β-tubulins class IIA (NP_0010606.1), IIB (NP_8211080.1), III (NP_006077.2), IVB (NP_006079.1), and V (NP_115914.1), with tubulin class I (NP_821133.1) scoring the most hits but lacking any unique peptides (data not shown). Thus, we conclude that there is no absolute specificity for β-tubulin isoforms in the trimer purified from HEK cells. This is in agreement with results from Cowan and co-workers (1) failing to find specificity for β-tubulin isoforms in different microtubules. Note that because the ratios of tubulins expressed in HEK cells are unknown, we cannot exclude the possible enrichment or preference of one isoform over another. Thus, TBCD appears to bind and co-purify with αβ-tubulin heterodimers in the absence of ARL2 but with only β-tubulin when ARL2 is present.

To further confirm the composition of our TBCD-ARL2-β-tubulin complex, we removed the GST tag and analyzed the purified protein by BN-PAGE. Coomassie staining of the protein (Fig. 4A, right lane) revealed a sharp band just below the 242-kDa size marker, consistent with the band observed in cell and tissue lysates (Fig. 2) and in marked contrast to the TBCD-α-tubulin-β-tubulin trimer (Fig. 4A, left lane). We then performed immunoblot analysis with TBCD, ARL2, and β-tubulin antibodies after resolving the sample by BN-PAGE. Fig. 4B shows that all three proteins are present in the band at 200 kDa. We also note the presence of a more diffuse band at ~480 kDa that is observed by Coomassie staining and ARL2 and TBCD immunoblots of the purified TBCD-ARL2-β-tubulin complex. This band is consistently present but highly variable in its relative abundance and is perhaps a dimer of the trimer or even an oligomer of TBCD and ARL2, as β-tubulin has not been observed in this band. As a final test of the composition of the TBCD-ARL2-β-tubulin trimer migrating at 200 kDa, we performed a two-dimensional gel analysis using sequential BN- and SDS-PAGE. After resolving the purified protein by BN-PAGE (first dimension; Fig. 4A, right lane), the 200-kDa band was excised, and minced gel pieces were loaded into the well of an SDS-PAGE gel (second dimension). Coomassie staining of the SDS gel revealed three bands at the expected sizes and no other bands (Fig. 4C). We note that the sum of the molecular weights of TBCD (133 kDa), ARL2 (20 kDa), and β-tubulin (55 kDa) is 208 kDa and very close to our estimate of 200 kDa, based on the electrophoretic mobility of the endogenous complex in BN gels. Similarly, the TBCD-ARL2-β-tubulin trimer eluted from the Superdex S200 column in fractions consistent with its apparent molecular mass of 200 kDa in comparison with protein standards (data not shown). Although these data are all consistent with a 1:1:1 complex, we cannot rule out the presence of two molecules of ARL2 or even a heterogeneous population with stoichiometries of 1:2:1 and 1:0:1 (TBCD-ARL2-β-tubulin), although we consider the latter unlikely. We also consider it unlikely that β-tubulin is substoichiometric with TBCD, as the resulting difference in size should be evident in the BN-PAGE gels.

Because some differences have been noted between human and bovine TBCD (12, 13, 29) and their apparent affinities for tubulins, we also expressed and purified bovine GST-TBCD from HEK cells. The bovine protein behaved in all respects comparable with the human TBCD in terms of levels of expression and co-purification with other proteins, both when expressed alone or when co-expressed with human ARL2 (data not shown). Note that human and bovine ARL2 proteins are 100% identical.

Thus, using recombinant preparations of TBCD purified from HEK cells, we have demonstrated its ability to bind specifically to either the αβ-tubulin dimer or to ARL2 and β-tubulin. The latter trimer displays the same electrophoretic mobility in BN-PAGE as the native complex seen in human and mouse cells and tissues; therefore, we conclude that the TBCD-ARL2-β-tubulin trimer is present in a wide array of animal cells and is the predominant form of TBCD found in many of them.

TBCE Can Bind to the TBCD-ARL2-β-Tubulin Trimer or to TBCB-α-Tubulin—Because two different models for tubulin folding propose a central role for TBCE, acting in concert with TBCD (5, 27), we sought to extend our analyses of TBCD complexes using the HEK system. Thus, we next examined the effects of co-expression with TBCE, TBCB, and TBCC on complex assembly and stability. We obtained plasmids that direct the expression of untagged human TBCB, TBCB (a generous gift from Dr. Zubala, University of Cantabria, Spain), or TBCE. We also generated an expression vector analogous to that used
Purification of TBCD-ARL2-β-Tubulin

for GST-TBCD to direct expression of GST-TBCE as described under “Experimental Procedures.”

Expression of GST-TBCD, followed by affinity purification, yielded a single band migrating in SDS gels with an apparent molecular mass of ~85 kDa, appropriate for this 58-kDa protein fused to the ~28-kDa GST (Fig. 5, lane 5). GST-TBCD expressed to the highest level of any protein or combination of proteins tested in our laboratory, with a yield of up to 30 mg of purified protein from a liter of adherent HEK cells (~10⁷ cells). In comparison, after optimization of time of expression, DNA/PEI ratios, and other aspects of the expression and purification, we found that we could obtain ~1 mg and ~5 mg of the GST-TBCD-α-tubulin-β-tubulin and GST-TBCD-ARL2-β-tubulin complexes, respectively, from 1 liter of adherent HEK cells (50 15-cm plates). No tubulins or other proteins co-purified with GST-TBCE, as assessed by Coomassie Blue staining of the purified preparation (data not shown). In contrast, when TBCB was co-expressed with GST-TBCE, the affinity-purified preparation included GST-TBCE and faint bands migrating at ~28 kDa and 55 kDa that are consistent with TBCB and tubulin, respectively (Fig. 5, lane 5). Immunoblotting confirmed the presence of α- and absence of β-tubulin in this preparation (Fig. 5, bottom panels). The amounts of TCB and α-tubulin that co-purified with GST-TBCE were more variable and always substoichiometric, as judged by the intensity of Coomassie Blue staining. We have not performed extensive buffer optimization for this trimer or worked with it as much as the TBCD-ARL2-β-tubulin trimer, but it appears to be less stable to subunit dissociation, predicted to be contributing to the variability or inconsistencies observed. Thus, co-expression in HEK cells can recapitulate the formation of the TBCB-TBCE-α-tubulin trimer described earlier and characterized structurally and functionally after reconstitution from components by Serna et al. (33), although our preparation is not suitable for such structural analyses.

When we co-expressed GST-TBCD, ARL2, and (untagged) TBCE in the HEK system, we found that the TBCE specifically co-purified, resulting in a complex of TBCD-ARL2-β-tubulin-TBCE, again with no α-tubulin (Fig. 5, lane 4). Thus, when present at comparable amounts, the TBCB can bind to the TBCD-ARL2-β-tubulin trimer and does so independently of α-tubulin. In contrast, co-expression of TBCD and TBCE (without ARL2) resulted in the purification of only the TBCD-α-tubulin-β-tubulin complex described above (Fig. 5, lane 2). Thus, in this system, TBCE appears to require ARL2 and/or the absence of α-tubulin to bind TBCD (Fig. 5, lane 4). Although the TBCE in this novel complex appears to be substoichiometric based on Coomassie Blue staining, we note that we have generated a similar complex to that argued in Nithianantham et al. (27) to be the central scaffold involved in tubulin folding in yeast. However, in our case, and in contrast to that previous study, there is always β-tubulin present in the different TBCD complexes. Indeed, in each of the different combinations of protein co-expressions, summarized in Fig. 5, we have never found TBCD to purify without one or both tubulins.

Because the binding of TBCC to the TBCD-TBCE-α-tubulin-β-tubulin complex is proposed to release the fully folded tubulin dimer, we also tested for effects of co-expression of human TBCC with GST-TBCD and ARL2. TBCC co-expression had no discernible effect on the complexes purified when co-expressed with TBCD, TBCD + TBCE, TBCD + ARL2, or TBCD + ARL2 + TBCE (data not shown). TBCC also did not co-purify with any of the protein combinations despite being expressed in a soluble form in these cells.

In a separate series of experiments, we also attempted to reconstitute the TBCD-ARL2-β-tubulin trimer from purified components. Although β-tubulin has recently been shown to be stable as a monomer in solution (34), it was not available to us in large quantities. Thus, we used commercially available porcine brain tubulin (Cytoskeleton, catalog no. HTS03-A) as a source of the tubulin heterodimer. No combination of this tubulin preparation with recombinant, purified human ARL2 (from Escherichia coli or HEK cells) and human GST-TBCD (from HEK cells) yielded assembly into any complexes (data not shown). The finding that the GST-TBCD-α-tubulin-β-tubulin...
Purification of TBCD-ARL2-β-Tubulin

FIGURE 6. Identification of point mutations in ARL2 that compromise binding to TBCD. A, tagged ARL2-HA and different ARL2 mutants were co-expressed with GST-TBCD in HEK cells and purified at a small scale. The clarified lysates (S14) and purified products (PD) were analyzed by a 4–20% SDS-PAGE gel and immunoblotted for ARL2 to monitor levels of expression and association with TBCD, respectively. Co-expressions of GST-TBCD with ARL2-HA and either ARL2 or ARL2[F50A] are shown here as examples. B, the relative level of co-purification of each ARL2 mutant was quantified as described under “Experimental Procedures.” Error bars represent one standard deviation.

preparation used in these studies appears to be at least partially aggregated makes interpretation of these results quite limited. Because the 200-kDa complex was found to be the most abundant form of TBCD and ARL2 in a number of tissues and cell lines, was characterized here to be a very stable trimer containing β- but not α-tubulin, and was found able to bind TBCE when ARL2 was present, we believe that the TBCD-ARL2-β-tubulin trimer is likely to serve a central role as a scaffold for assembly of other cofactors, including TBCE, involved in tubulin biogenesis.

ARL2 Mutants That Lose TBCD Binding Have Reduced Ability to Alter Microtubule Densities—Both ARL2 and TBCD have multiple actions in mammalian cells. They are each implicated in tubulin folding and microtubule dynamics, which are expected to be sensitive to the levels of tubulin dimer in cells. This makes dissection of specific functions and interactions technically challenging. To begin to assess the biological actions of one or more complexes of ARL2 with TBCD, we designed a series of mutations in ARL2 that were each predicted to compromise binding to TBCD. No structural information is currently available for TBCD, so we instead based our mutants on residues shown by co-crystal structures to be involved in the trimer (data not shown). Because our data strongly suggest that TBCD is stabilized by ARL2 binding (Fig. 4A), we considered the possibility that even a mutant with weakened affinity would still bind and co-purify with TBCD absent an alternative. Therefore, we developed a novel “relative binding assay” in which GST-TBCD was co-expressed with ARL2 and each of the ARL2 mutants. To differentiate between the two expressed ARL2 proteins, we used C-terminal, HA-tagged ARL2 (ARL2-HA), as it migrates more slowly in SDS-PAGE and is readily distinguished from the untagged proteins. Thus, GST-TBCD was co-expressed with two different ARL2 constructs, purified as before, and analyzed by SDS-PAGE, followed by immunoblotting. Two examples are shown in Fig. 6A. In Fig. 6A, left panel, we compare the levels of expression of ARL2 and ARL2-HA in the soluble fraction of the cell lysate (S14, see “Experimental Procedures”) and after affinity enrichment/pull-down (PD). Clearly the presence of the C-terminal HA tag does not alter the level of expression or binding to GST-TBCD, as both ARL2 proteins are pulled down in equal amounts. In contrast, the F50A mutant was found to express to higher levels than wild-type ARL2 but was very poorly brought down with TBCD. To quantify the relative abilities of the different ARL2 mutants to co-purify with GST-TBCD, we immunoblotted the co-purifying ARL2 proteins and quantified by densitometry using infrared fluorescence scans from a LI-COR Odyssey imaging system as described under “Experimental Procedures.” Although most ARL2 mutants expressed to similar levels in HEK cells, the ARL2[T30N] and ARL2[F50A] mutants did not, with decreased and increased expression levels over the wild type, respectively. To account for any differences in protein expression, the soluble fractions (S14) from cell lysates were immunoblotted for ARL2 and the relative expression levels (Rexp) were determined for each mutant by densitometry as the ratio of ARL2-HA/ARL2 mutant. Similarly, the relative levels of ARL2 co-purifying with GST-TBCD (Rpur) were determined after small-scale purifications, again from scans of immunoblots (Fig. 6). Each of these values was determined for each mutant as averages from triplicates of independently transfected wells of cells. Finally, to quantify the effect of each mutant, the ratio of expression levels was divided by the ratio of purification levels (Rexp/Rpur), and these results were normalized to the final ratio obtained for ARL2-HA/ARL2 (Fig. 6B). A
result greater than 1 indicates that the mutant bound preferably to ARL2-HA, whereas a value of less than 1 suggests that the mutant was less effective than ARL2-HA in binding to GST-TBCD. The results from this analysis reveal that the dominant activating mutant, ARL2[Q70L], binds comparably to ARL2 and ARL2-HA, whereas three of the other mutants tested ([H9004]1–9, L3A, and F50A) displayed markedly decreased binding to GST-TBCD (0.02 [H11006]0.01, 0.09 [H11006]0.1, and 0.04 [H250]0.00, respectively). The I6R mutant bound more than these three but was still only about half as effective ARL2-HA. Interestingly, the dominant inactivating mutant, ARL2[T30N], showed about a 5-fold increase in co-purification compared with ARL2-HA. This result is consistent with previously published data that found this mutant to be more effective at reversing the effects of TBCD expression on microtubule levels in cells (31). Thus, the two point mutants, L3A or F50A, or the nine-residue N-terminal truncation mutant were found to be clearly compromised in the ability to bind TBCD in this assay.

Expression of ARL2[Q70L] causes the loss of polymerized microtubules in cultured cells (37) (Fig. 7A). We reasoned that combining this activating mutation with one that compromises binding to TBCD would give us correlative evidence to assess whether these two processes, ARL2 binding to TBCD and regulation of microtubule densities, are linked. We combined the

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**FIGURE 7.** ARL2 double mutants reverse the microtubule loss phenotype caused by ARL2[Q70L] expression. A, HeLa cells were transiently transfected with plasmids directing the expression of ARL2 or point mutants, fixed 3 days later, and stained for ARL2 and α-tubulin. Transfected cells were identified by increased ARL2 staining (data not shown) and are indicated with asterisks. Note the loss of the microtubule array in the one cell expressing ARL2[Q70L] in the top right panel but its retention in others. B, representative images of cells stained with α-tubulin and scored as having normal microtubule density, intermediate microtubule loss, and severe microtubule loss, as described under “Experimental Procedures.” C, clarified lysates of the cells shown in A were immunoblotted (IB) for ARL2 to show relative levels of expression, with actin serving as a loading control. D, the effect of each ARL2 construct on microtubule density was scored using the classifications highlighted in B. Error bars represent standard deviation, and the average of three independent experiments is shown, with n ≥ 200 cells/condition. Scale bars = 10 μm.
loss-of-binding mutants with ARL2[Q70L] to generate the following double mutants: ARL2[L3A,Q70L], ARL2[I6R,Q70L], and ARL2[F50A,Q70L]. We expressed these mutants as well as ARL2 and ARL2[Q70L] in HeLa cells and performed immunocytochemistry with ARL2 and α-tubulin antibodies to mark transfected cells and microtubule arrays, respectively (Fig. 7A). To quantify the effects, microtubule densities were scored in three categories: normal, intermediate loss, and severe loss (examples of each of these are shown in Fig. 7B). Cells from each population were also immunoblotted with antibodies specific to ARL2 to compare relative expression levels of the ARL2 constructs (Fig. 7C). We note that the expression level of each double mutant is higher than the ARL2[Q70L] positive control. Therefore, the varying expression levels of the ARL2 constructs are not expected to dramatically affect the results of the assay. Fig. 7D summarizes the quantified effects of the ARL2 mutants on microtubule densities. Consistent with earlier studies, cells expressing ARL2[Q70L] showed a dramatic loss in polymerized microtubules, with ~75% of transfected cells displaying at least partial loss of microtubule arrays (Fig. 7D). However, cells expressing ARL2[L3A,Q70L] or ARL2[F50A,Q70L] showed a reversal of this microtubule loss phenotype, with <10% and <20%, respectively, of transfected cells showing only intermediate loss in microtubule densities. In contrast, the addition of the I6R mutation to Q70L did not reverse the effects of the dominant mutant on microtubule densities. Rather, this mutation, which showed only partial loss in relative affinity for TBCD (Fig. 6B), displayed only a slight increase in the percentage of cells displaying the intermediate phenotype (Fig. 7D). Thus, these results show a correlation between the ability of ARL2 and ARL2 mutants to bind TBCD and to alter microtubule dynamics in a cell-based assay, although, not surprisingly, the two assays are not quantitatively comparable.

Discussion

We set out years ago to examine the roles of ARL2 and TBCD in the αβ-tubulin biosynthesis and microtubule destruction pathways. We had previously purified ARL2 from bovine tissues and found it to co-purify with TBCD, but the heterodimer was inactive in tubulin folding or GTPγS binding assays (25). In contrast, Cowan and co-workers (31) had found that ARL2 inhibits the binding of TBCD to αβ-tubulin in their in vitro system, essentially competing for the binding of β-tubulin to TBCD and preventing TBCD from dissociating the αβ-tubulin heterodimer, which they termed the tubulin destruction pathway. We began here by monitoring the quaternary state of ARL2 and TBCD in cytosol from cultured mammalian cells and mouse tissues and found several cell lines and tissues in which the clear majority of each protein co-fractionated in both native polyacrylamide gels and gel filtration medium, although this was also quite variable between tissues and even species. When TBCD and ARL2 were overexpressed in HEK cells and affinity-purified, β-tubulin was shown to co-purify in a novel TBCD-ARL2-β-tubulin trimeric complex with an estimated 1:1:1 stoichiometry. The characterization of this and related complexes revealed several features in common with results from Cowan and co-workers (29, 31) but also some clear differences that ultimately lead us to different conclusions regarding the likely roles of ARL2 in tubulin biology and the nature of the complex(es) in which it acts. Most importantly, we find that every time we purify TBCD from cultured human cells, it co-purifies with at least one tubulin. Additionally, when ARL2 is present in sufficient amounts, the complex includes ARL2, resulting in a more stable and homogeneous complex. TBCE can bind to the TBCD-ARL2-β-tubulin complex, even in the absence of α-tubulin. We discuss below the novel protein complexes described here and how they are thought to relate to previously described complexes.

We found the predominant form of both ARL2 and TBCD in many cells and tissues to be a complex of ~200 kDa that co-migrates in native gels and co-purified from HEK cells as an apparent 1:1:1 complex of TBCD-ARL2-β-tubulin. This trimer has not been described before, although we speculate that it was present in previous studies (31). The assays developed by Cowan and co-workers (5, 6) that identified the five tubulin co-chaperones, or cofactors A–E, used low-percentage (8%) polyacrylamide gels to assess protein purity. At 20 kDa, ARL2 runs at the dye front in such gels and was likely simply not appreciated to be a partner of TBCD. This conclusion is supported by our observations that we have never found a preparation of TBCD that is monodisperse and stable that lacks ARL2. Thus, we believe the “middle band” described by Tian et al. (5) that was identified by addition of TBCD to in vitro translated β-tubulin and migrated in native gels with a size of 200–300 kDa contains ARL2 in addition to the TBCD and β-tubulin described. And later, when ARL2 was found to bind to TBCD, they found that addition of TBCD to in vitro translated ARL2 resulted in the formation of an ~200-kDa complex that was shown to contain ARL2 and TBCD but was not analyzed for β-tubulin (31). The consistent observations in multiple laboratories that gel filtration or native gels yield apparent molecular masses for TBCD in the range of 200–300 kDa has been interpreted as possible homodimerization, but we believe it is the result of a heterotrimer that actually migrates very close to its expected size of 208 kDa. Thus, the TBCD-ARL2-β-tubulin trimer is a stable component in a wide array of mammalian cells and tissues that we have purified in milligram amounts from HEK cells and that we speculate was present and functional in the tubulin folding assays that first identified the cofactors. This is consistent with our conclusion that TBCD-ARL2-β-tubulin is an intermediate in the folding reaction. Indeed, based on our reinterpretation of the pioneering work of Cowan and co-workers (5, 6, 31), we propose that it is the central scaffold onto which α-tubulin is later added, in concert with TBCE and TBCB.

We also found that, in the absence of ARL2, TBCD bound and co-purified with αβ-tubulin, generating the TBCD-α-tubulin-β-tubulin trimer, although, as purified from HEK cells, this is not stable as a monodisperse species. Addition of purified ARL2, either from bacteria or HEK cells after overexpression, to this trimer had no effect, i.e. it did not bind stably or alter the amount or ratio of tubulins bound. This is in apparent contradiction to work described by Bhamidipati et al. (31) in which addition of TBCD to in vitro translated tubulin dimers resulted in the formation of a new band in native gels that is thought to be a dimer of TBCD-β-tubulin, although α-tubulin was not
monitored in that study. Addition of ARL2 in that system decreased the amount of tubulin dimers binding to TBCD, which was interpreted as ARL2 competing for the binding of TBCD and β-tubulin. They even speculate that the appearance of another band, present only when excess ARL2 was added, was the TBCD-ARL2-β-tubulin trimer. In contrast, we have never observed evidence of a TBCD-ARL2 or TBCD-β-tubulin dimer in or coming from the HEK cell expression system. Trivial explanations for these differences might be found in the different gel systems, protein preparations, or assays used. But another model to explain these apparent differences is that TBCD can bind to tubulin dimers, perhaps even in both dimeric and polymerized (microtubule) states. Thus, the band generated upon addition of TBCD to in vitro translated αβ-tubulin dimers could be the same as our TBCD-α-tubulin-β-tubulin trimer, which, in Bhamidipati et al. (31), is partially converted to the TBCD-ARL2-β-tubulin trimer, the extra band they see in excess ARL2.

Our ability to purify the TBCD-α-tubulin-β-tubulin trimer, even though not monodisperse, is compelling evidence of a specific interaction between TBCD and tubulin heterodimers. This specific trimer of TBCD-α-tubulin-β-tubulin might result in cells from 1) residual TBCD binding to recently folded αβ-tubulin based on the Cowan model of folding (i.e. slow release or resolution of a larger complex involved in the final step of folding), 2) TBCD binding to previously folded, native αβ-tubulin (perhaps the “tubulin destruction” pathway), or 3) TBCD binding to microtubules with resulting loss of microtubule integrity and at least a quasi-stable trimer. We cannot currently distinguish between these possibilities. However, because overexpression of TBCD results in the loss of microtubules in cells (12, 31, 32) we believe option 3 to be the most likely source of this trimer from cells. More work is clearly needed to provide stronger testing of these models.

Prior to this study, there have been two different complexes of ARL2 and TBCD that have been purified. One of these complexes was identified in a previous study from our laboratory by following ARL2 through several chromatographic steps using bovine tissues as the source (25). This produced a complex of ARL2, TBCD, and protein phosphatase 2A, although we now believe this to be coincidental co-purification of PP2A. Notably, there was no evidence of tubulin in this preparation. A recent study by Nithianantham et al. (27) showed that co-expression and purification of yeast orthologs of TBCD, TBE, and ARL2 (CIN1p, PAC2p and CIN4p, respectively) from bacteria resulted in the formation of the orthologous TBCD-TBCE-ARL2 complex. As this complex was purified from bacteria, there was no opportunity for co-purification of tubulin. However, they showed that purified tubulin heterodimer could bind the complex in vitro. Although both of these approaches to purifying TBCD-ARL2 complexes have merit and are predicted to produce biologically active complexes, the methods used in this study allowed us to identify the predominant form of ARL2 and TBCD in many mammalian cells (i.e. TBCD-ARL2-β-tubulin) and generate milligram amounts of the purified complex. Based on the ability to add other tubulin cofactors to this complex, namely TBCE and its predominance in a number of tissues and cell lines, we posit that the TBCD-ARL2-β-tubulin trimer is a key scaffold onto which other proteins bind in the tubulin biogenesis pathway. Admittedly, further characterization of these ARL2-cofactor complexes will be required to continue the development of a more complete model of tubulin folding and the effects of TBCD and ARL2 on tubulin pools and microtubule arrays.

The identification of ARL2 mutants that disrupt interaction with TBCD and, thus, formation of the TBCD-ARL2-β-tubulin complex has allowed us to test the biological relevance of the ARL2-TBCD interaction using a cell-based assay in correlation with a novel ARL2-TBCD binding assay. Combining these mutants (L3A, I6R, and F50A) with the dominant activating ARL2[Q70L], which causes wholesale loss of microtubules, revealed a good correlation between loss of TBCD binding and loss of effects on microtubules. ARL2[Q70L] is considered constitutively active because of its inability to hydrolyze GTP. As the relative binding assay shows, this mutant binds to TBCD at similar levels as wild-type ARL2. Thus, when ARL2[Q70L] is expressed in cells at severalfold higher levels than endogenous ARL2 we believe that ARL2[Q70L] is incorporated into the TBCD complex in place of the endogenous protein. The resulting loss of polymerized microtubules may then result from the inability of ARL2[Q70L] to cycle between GTP- and GDP-bound conformational states as a result of the absence of the glutamine that is directly involved in hydrolysis of the γ-phosphate. This interesting but unproven model clearly places conformational changes occurring in ARL2 as an important component in the effects on microtubules in cells and should be examined further in later studies. When the loss-of-binding double mutants (L3A or F50A with Q70L) are expressed, they are unable to cause loss of microtubules, presumably because of their relative inability to complex with TBCD. Therefore, it seems that not only the binding of ARL2 to TBCD-β-tubulin is important but that the hydrolysis of guanine nucleotide on the bound ARL2 is required for proper microtubule regulation. Indeed, in a related study,3 we show that ARL2, but not β-tubulin, binds guanine nucleotides in the TBCD-ARL2-β-tubulin trimer.

The use of second site mutations combined with a dominant activating (Q70L) mutation of ARL2 has also proven to be invaluable in cleanly dissecting the roles of ARL2 in regulating tubulin folding/microtubule density from those inside mitochondria; i.e. regulating mitochondrial fusion from the intermembrane space.4 Although not important to this study, about 5–10% of cellular ARL2 is found inside mitochondria (38), and when the ARL2[Q70L] mutant is expressed, mitochondria become hyperfused/elongated.5 When the additional mutations used here were tested for effects on mitochondria, we found that ARL2[L3A,Q70L], ARL2[I6R,Q70L], and ARL2[F50A,Q70L] each retained full activity in mitochondrial fusion despite the complete or nearly complete loss of binding to TBCD (Fig. 6) or effects on microtubules (Fig. 7). Because of its multiple cellular functions, such mutational analyses are

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3 J. W. Francis, D. Goswami, S. J. Novick, P. R. Griffin, and R. A. Kahn, manuscript in preparation.
4 L. E. Newman, C. R. Schiavon, R. E. Turn, and R. A. Kahn, submitted for publication.
predicted to be increasingly important in deconvoluting the complexities of signaling by this one essential, regulatory GTPase.

Because the design of the ARL2 mutants was based on crystal structures of ARL2 bound to other effectors, it is almost certain that expression of these mutants in cells may also disrupt other ARL2 interactions. However, of the known ARL2 binding proteins, TBCD is the only one that has an integrated role in the production of αβ-tubulin heterodimer and in the regulation of microtubule stability. Thus, we conclude that the effect of the ARL2 mutants on polymerized microtubules is specifically due to the interaction between ARL2 and TBCD and, ultimately, the formation of the TBCD-ARL2-β-tubulin complex.

Further evidence of the importance of the TBCD-ARL2-β-tubulin complex has been recently highlighted by Flex et al. (17), in which several point mutations in TBCD were described that lead to neurodegenerative disorders in humans. When a subset of the identified TBCD mutants were co-expressed with ARL2 in our HEK cell system, they result in a reduced amount of β-tubulin in the TBCD-ARL2-β-tubulin complex. Additionally, the purified complexes each have reduced stability (lower melting point, $T_m$) in a thermal denaturation assay (17). The study also analyzed patient cells in an in vivo tubulin polymerization (nocodazole washout) assay. The TBCD mutants that had reduced binding to β-tubulin showed an increase in the rate of tubulin polymerization compared with control cells. Similar studies have recently added to our appreciation of the importance of TBCD and its binding to ARL2 and β-tubulin in related neurological syndromes (18, 19, 20, 39). Together, these findings argue that the TBCD-ARL2-β-tubulin complex is vital for the proper homeostasis that is required in microtubule dynamics and human health.

In addition to the very stable trimer of TBCD-ARL2-β-tubulin, we also purified from HEK cells the tetramer of TBCD-ARL2-β-tubulin-TBCE, although the stoichiometry of the TBCE in the complex was more variable and lower than those of the other three components. The functional significance of this complex is difficult to evaluate at this point but could be used to identify sites in the other three proteins to which TBCE binds. It also suggests that α-tubulin is not required for binding of TBCE to TBCD-containing complexes, although it is possible that its presence may increase the affinity. The finding that the HEK cell system could also confirm the clinical importances of this complex will prove to be beneficial to both the scientific and clinical communities.

Experimental Procedures

Cell Culture—Human cervical carcinoma (HeLa), HEK293T/17, mouse N2A neuroblastoma, mouse embryonic fibroblast (NIH-3T3), and human neuroblastoma (SH-SY5Y) cells were obtained from the ATCC and grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM glutamine at 37 °C in the presence of 5% CO$_2$. Cells were screened for mycoplasma regularly by staining with Hoechst 33342 DNA dye, usually in conjunction with immunofluorescence experiments (described below).

Plasmids—Plasmids directing expression of human ARL2, ARL2[T30N], or ARL2[Q70L] used pcDNA3.1 as a vector, as described previously (37), and were used as starting points to generate plasmids used for expression of the following mutants through PCR-based mutagenesis: ARL2[L3A], ARL2[L6R], ARL2[F50A], ARL2[L3A,Q70L], ARL2[L6R,Q70L], ARL2[F50A,Q70L], ARL2-HA, and HA-ARL2. A plasmid containing human TBCD (NP_005984.3) was obtained from the ATCC (clone MGC-1583), and the full-length open reading frame was moved into the pLEXm-GST vector (a gift from James Hurley, National Institutes of Health) at NotI and XhoI sites to generate an N-terminal, GST-tagged construct (GST-TBCD). Full-length bovine TBCE was cloned into the same sites. The construct has a TEV protease cleavage site between the GST and TBCD that results in a 12-amino acid residual extension at the N terminus of TBCD after cleavage. A plasmid containing the full-length open reading frame of human TBCD was purchased from the ATCC (catalog no. MGC-8912, lot 61980096) and directs the expression of isofrom A (NP_003184.1). TBCE was also cloned into the pLEXm vector using SacI and SphI sites, inserted with PCR, to generate pLEXm-GST-TBCE. Plasmids directing the expression of human TBCB or TBCC were generous gifts from Juan Carlos Zabala (University of Cantabria, Madrid, Spain) and were both in pcDNA3.1.

Antibodies—Affinity-purified rabbit polyclonal ARL2 (R-86336) antibodies were prepared as described by Sharer and Kahn (38). Affinity-purified rabbit polyclonal TBCD antibodies were prepared as described by Cunningham and Kahn (12). As these two rabbit polyclonal antibodies were generated in our laboratory and purified whole protein antigens are readily available, we routinely perform antigen competition experiments to confirm specificity of signals in both immunoblot and immunofluorescence experiments. Other antibodies used in this study included a rabbit polyclonal GST (Sigma, catalog no. G7781), mouse monoclonal actin (Sigma, catalog no. A3853), mouse monoclonal α-tubulin (Sigma, clone DM1A, catalog no. T9026), and mouse monoclonal β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa, catalog no. E7-c).

Cell Transfections—For immunofluorescence experiments, cells were transfected at ≥90% density in 6-well plates. The numbers given here are for transfection of a single well. The amounts of DNA and Lipofectamine were separately optimized for ARL2 expression in HeLa cells. A ratio of 2 μg:1 μg (Lipofectamine:DNA) yielded the highest transfection efficiency. ARL2 plasmids (2 μg) were diluted in 250 μl of OptiMEM (Invit-
Purification of TBCD-ARL2-β-Tubulin

For recombinant protein purifications, HEK cells were transfected at ~70%–90% density using PEI (Polysciences, catalog no. 24765-2) at a 1:3 ratio (DNA:PEI). In all transfections and cotransfections, plasmids were used at 1 µg of DNA/ml of medium and scaled accordingly for larger/smaller transfections. Numbers given here are for one 10-cm plate (~10⁷ cells). Prior to transfection, DNA and PEI were mixed in 1 ml of serum-free medium and incubated at room temperature for 15 min. Cell culture medium was changed to 9 ml of a reduced-serum medium (DMEM + 2% FBS), and the transfection mixture was added dropwise to the cells (10-ml final volume). After 24 h, cells were trypsinized and replated onto coverslips, typically at a 1:4 split. Cells were allowed to attach overnight before fixation. This transfection typically resulted in ~70% of cells expressing exogenous ARL2 and ~50% expressing ARL2[T30N] or ARL2[Q70L] when assayed 48 h after the start of transfection.

Preparation of Cell/Tissue Lysates—Cells were grown to confluence before collecting in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ (pH 7.4)). Cell pellets were resuspended in lysis buffer (0.5 ml/10⁷ cells; 25 mM HEPES, 100 mM NaCl, 1 mM DTT, and 1% CHAPS (pH 7.4)) and incubated at 4 °C for 15 min with gentle rotation. The lysates were clarified at 14,000 g for 15 min. For large pellets, a Dounce homogenizer prior to centrifugation to generate a homogenous lysate was necessary. Mouse tissues were collected from freshly euthanized animals, minced with a razor blade, and resuspended in lysis buffer, and then cells were lysed using a Dounce homogenizer prior to centrifugation to generate the soluble fraction (S100). Bovine and human brain samples were obtained from frozen tissue stocks and similarly prepared to generate the S100.

Gel Electrophoresis—BN-PAGE was performed using 4–16% Novex BisTris gels (Invitrogen, catalog no. BN1002) according to the instructions of the manufacturer. Native protein standards (Invitrogen, catalog no. LC0725) were used as references and to estimate sizes of protein complexes. Denaturing SDS-PAGE was performed using 13% or 4–20% (Bio-Rad, catalog no. 4561096) acrylamide gels with Precision Plus Protein All Blue Standards (Bio-Rad, catalog no. 1610373). For each ARL2 mutant tested, the S14 sample (15 µl of slurry) and the supernatant of the S14 sample (15 µl of slurry) were equilibrated with lysis buffer, and 100 µl of slurry (~50 µl of beads)/10-cm plate was added to the S14 and incubated overnight at 4 °C with gentle rotation. The sample was then added to a Poly-Prep chromatography column (Bio-Rad, catalog no. 7311550) and filtered by gravity. The column was washed with 5 column volumes of wash buffer (25 mM HEPES, 100 mM NaCl, and 1 mM DTT (pH 7.4)). The beads were then resuspended in 1 column volume of elution buffer (25 mM HEPES, 100 mM NaCl, 1 mM DTT, and 20 µl glutathione (pH 8.0)) and incubated at 4 °C for 20 min before elution of the sample. Another column volume of elution buffer was added to the beads and immediately collected to maximize recovery. Eluted protein concentration was determined using a Bradford assay. In some instances, TEV protease was added to the sample at 1% (w/w) the amount of purified protein and allowed to incubate at 4 °C for at least 4 h and overnight. The cleaved sample was further purified by gel filtration over a Superdex 200 column (Pharmacia), and the fractions were analyzed in SDS-PAGE gels. Appropriate fractions were pooled and concentrated by ultrafiltration as needed for use.

Alternatively, small-scale batch purifications of GST-tagged proteins were performed to allow analyses of protein complexes to be performed in a more high-throughput manner. Cells were transfected using PEI, as described above, in a 6-well plate, and cell pellets were resuspended in 200 µl of lysis buffer per well (~10⁷ cells). After equilibration, 10 µl of glutathione-conjugated Sepharose beads (20 µl of slurry) was added to the S14 of each sample and incubated overnight at 4 °C with gentle rotation. The samples were spun at 14,000 x g for 30 s to pellet the beads, and the supernatant was removed by careful aspiration. The beads were washed three times by adding 500 µl of wash buffer and immediate spinning at 14,000 x g for 30 s. After aspiration of the last wash, 20 µl of 2× SDS sample buffer was added directly to the beads, and samples were heated at 95 °C for 5 min. For Coomassie staining, 15 µl of each sample was loaded onto the gel. For immunoblotting, the samples were diluted 1:10 in 1× SDS sample buffer, and 15 µl was loaded onto the gel.

Relative Binding Assay—To quantify the effects of ARL2 mutants on interaction with TBCD, a relative binding assay was developed. GST-TBCD was co-expressed in HEK cells with C-terminally tagged ARL2-2HA and an untagged ARL2 mutant. Batch purification was performed as described above. During purification, a sample of S14 was collected, diluted 1:10 in wash buffer, and then diluted again (1:1) in 2× SDS sample buffer. For each ARL2 mutant tested, the S14 sample (15 µl) was loaded adjacent to the final product of the pulldown (15 µl, as described above). The samples were resolved by SDS-PAGE (4–20% acrylamide) and immunoblotted with ARL2 antibodies. A fluorescent secondary antibody (Invitrogen, catalog no. A11374) was used, and the blots were imaged with an Odyssey imaging system (LI-COR). To account for differing levels of expression in the assay, the level of expression of ARL2-2HA, as determined by densitometry, was compared with the expression level of the ARL2 mutant by taking the ratio of ARL2-2HA: ARL2 mutant in the S14 sample (Rexp). The relative levels of ARL2-2HA and ARL2 mutant co-purifying with GST-TBCD were similarly determined in the PD sample (Rpur). The ratio of expression levels was then divided by the ratio of co-purifica-
Purification of TBCD-ARL2-β-Tubulin

tion levels (R_{exp}/R_{pur}), and these results were normalized to those obtained for ARL2-HA and wild-type ARL2 expression and co-purification with GST-TBCD. A final result greater than 1 indicates that the ARL2 mutant co-purified with GST-TBCD better than wild-type ARL2. A value less than 1 indicates that the mutant co-purified worse than the wild type, and a value equal to 1 implies that the mutant bound and co-purified similar to the wild type.

**Immunofluorescence**—Cells were grown on coverslips coated with Matrigel (BD Biosciences, catalog no. 356231). Cells were fixed in a prewarmed (37 °C) solution of 4% paraformaldehyde in PBS (v/v) and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. Incubation with primary antibodies was carried out in filtered PBS containing 1% (w/v) bovine serum albumin at 4 °C overnight, followed by four 5-min washes in PBS. Secondary antibodies (1:500, Alexa fluorophores, Invitrogen) were incubated in the same buffer for 1 h at room temperature, followed by two 5-min washes in PBS. DNA was stained with Hoechst 33342 for 4 min, followed by two 5-min washes in PBS prior to mounting onto slides using ProLong Antifade (Invitrogen). Images were acquired using an Olympus FV1000 microscope and Olympus Fluoview v1.7 software with 488 and 543 laser excitation and a 100× oil objective (1.45 numerical aperture (NA)). Z stacks were acquired with a step size of 0.37 μm and converted to average image intensity projections using ImageJ where indicated.

The effects of protein expression on microtubule morphology were scored by visual inspection of fixed, stained HeLa cells. To quantify consequences of protein expression on microtubule density, we used the following categories as described previously (37): complete loss, near or complete loss of microtubules; intermediate loss, microtubule density is obviously lower than untransfected cells in the same field; normal, transfected cell cannot be readily distinguished from untransfected cell in terms of microtubule density. A cell was excluded from analysis when it appeared to be dying, as defined by cell rounding with membrane blebs and fragmented nuclear staining; these cells typically had the highest levels of expression in the cell population. Only transfected cells were scored, which were identified by ARL2 immunofluorescence signal (ARL2 signal obviously brighter than the surrounding cells in the field).

**Reproducibility and Statistical Analyses**—Every experiment described has been independently repeated at least twice. Protein preparations were performed multiple times, and variations in composition or relative abundance of components are indicated in the text. For quantification of immunofluorescence assays, experiments were independently repeated at least three times, and at least 200 cells per condition were analyzed per experiment. For quantification of the relative binding assay (see above), averages are the result of three independent experiments. Error bars for both sets of quantified data represent one standard deviation.

**Author Contributions**—J. W. F. conducted most of the experiments, analyzed the data, and wrote the paper. L. E. N. designed, performed, and analyzed the experiments shown in Fig. 7, A, B, and D. I. A. C. designed, performed, and analyzed the experiments shown in Fig. 1. R. A. K. coordinated the study and wrote the paper with J. W. F.

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4348 JOURNAL OF BIOLOGICAL CHEMISTRY
