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Effect of TBCD and its Regulatory Interactor Arl2 on Tubulin and Microtubule Integrity

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Abstract

Assembly of the α/β tubulin heterodimer requires the participation of a series of chaperone proteins (TBCA-E) that function downstream of the cytosolic chaperonin, CCT, as a heterodimer assembly machine. TBCD and TBCE are also capable of acting in a reverse reaction in which they disrupt native heterodimers. Homologs of TBCA-E exist in all eukaryotes, and the amino acid sequences of α- and β-tubulin isotypes are rigidly conserved among vertebrates. However, the efficiency with which TBCD effects tubulin disruption *in vivo* depends on its origin: bovine (but not human) TBCD efficiently destroys tubulin and microtubules upon overexpression in cultured cells. Here we show that recombinant bovine TBCD is produced in HeLa cells as a stoichiometric co-complex with β-tubulin, consistent with its behavior *in vitro* and *in vivo*. In contrast, expression of human TBCD using the same host/vector system results in the generation of TBCD that is not complexed with β-tubulin. We show that recombinant human TBCD functions indistinguishably from its non-recombinant bovine counterpart in *in vitro* CCT-driven folding reactions, in tubulin disruption reactions, and in tubulin GAP assays in which TBCD and TBCC stimulate GTP hydrolysis by β-tubulin at a heterodimer concentration far below that required for polymerization into microtubules. We conclude that bovine and human TBCD have functionally identical roles in *de novo* tubulin heterodimer assembly, and show that the inability of human TBCD to disrupt microtubule integrity upon overexpression *in vivo* can be overcome by siRNA-mediated suppression of expression of the TBCD regulator Arl2 (ADP Ribosylation Factor-like Protein 2).

Keywords

Tubulin; Tubulin Folding; Heterodimer Assembly; Microtubules; Tubulin Specific Chaperones; Arl2

INTRODUCTION

Microtubules are ubiquitous and dynamic polarized polymers assembled from α/β tubulin heterodimers. They participate in a broad range of both essential and specialized cellular functions. These include the determination of cell shape as part of the interphase cytoskeleton; cell division as the principal component of the mitotic and meiotic spindle; intracellular transport as the tracks that direct the movement of organelles and other cellular components; and cell motility as a key element of cilia and flagella.

Classical experiments have established that the information required for all proteins to acquire their correct three-dimensional structure is contained within their linear sequence of amino acids (Anfinsen 1973). However, the conditions that prevail within eukaryotic cells –

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including a very high local protein concentration, as well as molecular crowding effects and a relatively high temperature – frequently hamper the efficient attainment of a protein's native conformation. Not surprisingly, therefore, a class of proteins termed chaperones has evolved whose function is to prevent or reduce the occurrence of off-pathway folding events that would otherwise lead to intracellular misfolding and aggregation (Hartl and Hayer-Hartl 2009). Amongst these, chaperonins are a class of multi-subunit ATP-dependent chaperones that provide a sequestered chamber within which newly synthesized or misfolded proteins can partition to the native state in the absence of intermolecular interactions that might otherwise lead to improper folding. In *E. coli*, the prototypical and well-studied chaperonin GroEL exists as a stacked back-to-back tetradecameric double-ring structure assembled from a single 57 kDa polypeptide which functions in conjunction with its co-chaperonin GroES (Hartl 1996; Hartl and Hayer-Hartl 2002; Horwich et al. 2007). In the cytosol of eukaryotes, the distantly related chaperonin homolog of GroEL is CCT (cytosolic chaperonin containing T-complex polypeptide 1) (Horwich et al. 2007). CCT consists of eight or nine member rings in which each polypeptide is encoded by a different gene and is arranged in a defined orientation. In contrast to GroEL, CCT functions independently of a co-chaperonin, and is thought to participate in the facilitated folding of a range of eukaryotic proteins, principal among which are the actins and tubulins (Dekker et al. 2008). Indeed, the participation of CCT is essential for the productive folding of these cytoskeletal proteins (Vinh and Drubin 1994; Ursic et al. 1994; Cowan and Lewis 2002; Young et al. 2004).

Although actins can partition to the native state via ATP-dependent interaction with CCT alone, this is not the case with respect to the α/β tubulin heterodimer. Rather, heterodimer assembly requires the additional participation of a series of tubulin-specific chaperone proteins (TBCA-E) that function together as a heterodimer assembly machine (Cowan and Lewis 2002; Szymanski 2002). A model summarizing our current understanding of the tubulin heterodimer assembly pathway is shown in Fig. 1. Nascent α- and β-tubulin polypeptides are protected from off-pathway folding pathways by interaction with the cytosolic chaperonin, CCT (Gao et al. 1992). One or more cycles of ATP-dependent interaction with CCT leads to the production of quasi-native tubulin folding intermediates, defined by the incorporation of GTP. These intermediates are captured upon discharge from CCT either by TBCA (in the case of β-tubulin) or TBCB (in the case of α-tubulin). TBCA/β-tubulin and TBCB α-tubulin transfer their respective target proteins to TBCD and TBCE. These two complexes (i.e. TBCD/β-tubulin and TBCE/α-tubulin) converge to form a supercomplex (TBCD/β/TBCE/α). Entry of TBCC into this supercomplex triggers the hydrolysis of GTP in the β-tubulin E-site. This reaction acts as a switch for the release of the newly formed α/β-tubulin heterodimer, which is then competent for GTP exchange and polymerization into microtubules. The folding reaction is reversible: TBCD and TBCE are capable of interacting with the native heterodimer *in vitro*, sequestering the β- and α-subunit, respectively (the so-called back-reaction, shown as dashed lines in the Figure) (Tian et al. 1997; Cowan and Lewis 2002). Consistent with these data, overexpression of human TBCE or bovine TBCD *in vivo* results in tubulin destruction and microtubule obliteration (Bhamidipati et al. 2000). The ability of TBCD to interact with β-tubulin is regulated via its interaction with the small GTPase Arl2 (ADP Ribosylation Factor Like Protein 2), to which it binds *in vivo* (Bhamidipati et al. 2000) via the formation of an Arl2/TBCD/PP2A (Protein Phosphatase 2) co-complex (Shern et al. 2003). This reaction is thought to serve a regulatory function (Bhamidipati et al. 2000).

TBCD was originally discovered via its purification as a 120 kDa protein from bovine testis tissue that was responsible for forming a characteristic co-complex with β-tubulin in *in vitro* folding assays done with CCT in the presence of ATP and GTP (Tian et al. 1996). TBCD also contributes to tubulin GAP (GTPase Activating Protein) activity, in which TBCD and TBCE induce GTPase activity via hydrolysis of E-site GTP in the native tubulin heterodimer

at a concentration far below that required for polymerization into microtubules (Tian et al. 1999). In addition to its participation in *de novo* tubulin heterodimer assembly and tubulin GAP activity, recent evidence implicates TBCD in recruitment of the γ-tubulin ring complex at centrosomes and organization of the mitotic spindle (Cunningham and Kahn 2008; Fanarraga et al. 2010). The gene encoding TBCD is essential for life in higher eukaryotes, as shown by genetic experiments in the model organisms *S. pombe* and *A. thaliana* (Hirata et al. 1998; Radcliffe et al. 1999; Steinborn et al. 2002).

Curiously, the effect of overexpression of TBCD in cultured cells differs depending on the species of origin of the TBCD protein: bovine TBCD efficiently destroys tubulin and microtubules upon overexpression in HeLa cells (Bhamidipati et al. 2000), while overexpression of the human homolog does not (Cunningham and Kahn 2008). These observations are surprising given the rigid evolutionary conservation of α- and β-tubulin amino acid sequences among all vertebrate species, and the relatively high similarity of bovine and human TBCD. Here we describe the expression, purification, biochemical and biological properties of bovine and human TBCD produced via the use of adenovirus vectors. We show that the two recombinant proteins are produced in their (human) host cells as different molecular entities: in the case of bovine TBCD, the recombinant protein purifies as a co-complex with β-tubulin, while in the case of human TBCD, the recombinant protein purifies as a single molecular species. The latter protein functions in *in vitro* CCT-driven folding assays and in *in vitro* tubulin disruption reactions in a manner indistinguishable from its non-recombinant bovine counterpart. Moreover, the authentic bovine and recombinant human TBCD homologs function equally well in tubulin GAP assays (Tian et al. 1999). These data are consistent with TBCD from mammalian species having functionally identical roles in *de novo* tubulin heterodimer assembly. We show that the microtubules of HeLa cells are protected from human TBCD-mediated disruption via interaction of this protein with Arl2.

MATERIALS AND METHODS

Construction of Recombinant Adenoviruses

Full-length cDNAs encoding either bovine or human TBCD were generated by PCR as described (Tian et al. 1996) and checked by DNA sequencing. Recombinant adenoviruses engineered for the expression of bovine or human TBCD were generated by insertion of these cDNAs into the pShuttle-CMV vector. Recombinant viruses were prepared via homologous recombination in *E. coli* carrying the pAdEasy-1 plasmid using the AdEasy kit and amplified in HEK293AD cells following the manufacturer's instructions (Stratagene Inc., La Jolla, CA). HeLa cells were grown in DMEM supplemented with 10% fetalcalf serum and infected at an approximate multiplicity of infection (MOI) of 500 at about 90% confluence. In some experiments designed specifically to generate the recombinant protein in reduced yield (see text), the MOI was reduced to about 50. In all cases, cells were harvested by scraping 48 h post infection, recovered by centrifugation, washed in ice-cold PBS and used for the preparation of soluble extracts as described below.

Protein Purification

Authentic bovine TBCD was purified from testis tissue as described previously (Tian et al. 1996). For the purification of recombinant bovine and human TBCD, extracts of approximately 5×10^9 adenovirus infected HeLa cells were prepared by Dounce homogenization in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT containing a cocktail of protease inhibitors (Roche Applied Science Inc., Indianapolis, IN). Lysates were cleared of particulate material by successive centrifugation steps done at 4°C at 5,000, 30,000 and 200,000 x g. Cleared lysates were fractionated by FPLC (GE Healthcare,

Piscataway, NJ) on successive columns of Sepharose Q High Performance, hydroxylapatite, MonoQ and Sepharose 6 as described previously (Tian et al. 1996). In all dimensions, the recombinant proteins were detected either directly as a band with an apparent molecular mass of 120 kDa via staining with Coomassie blue, or indirectly via Western blotting using an antiserum against bovine TBCD (Tian et al. 1996) or raised against a hapten-conjugated synthetic polypeptide derived fromhuman TBCD (see below). The approximate yield and fold enrichment of these proteins at each step is summarized in Table 1.

Mass Spectrometry

Authentic and recombinant bovine TBCD were excised as Coomassie blue-stained bands from SDS-PAGE and reduced, alkylated with iodoacetamide and digested with trypsin in a ProGest workstation (Genomic Solutions, Cambridge, UK). Digestion products were analyzed by nano liquid chromatography tandem MS on a ThermoFisher LTQ Orbitrap XL.

In Vitro Folding and Tubulin GAP Assays

^{35}S-labeled β-tubulin target protein was expressed in *E. coli* and the inclusion bodies purified and unfolded in 8 M urea as described (Gao et al. 1992). *In vitro* folding assays were done in folding buffer containing CCT, ATP, GTP and TBCD as described previously and the reaction products resolved by electrophoresis on 4.5% native polyacrylamide gels (Tian et al. 1995; Tian et al. 1996; Tian et al. 1997). Tubulin GAP assays were performed using purified bovine brain tubulin, TBCC, TBCD, TBCE and γ-^{32}P-GTP as described (Tian et al. 1999).

Antisera

A human Arl2 sequence cloned as described (Bhamidipati et al. 2000) was inserted into thepET23 vector and used to express the recombinant protein following IPTG-induced expression in *E. coli* BL21(DE3) cells. Bacterial cells were lysed in 10 mM Tris-HCl, 1 mM EDTA pH 7.8 using a French Pressure Cell, and a particle-free soluble extract prepared by centrifugation at 200,000 x g. The recombinant protein was purified from this extract by anion exchange chromatography via FPLC using the following dimensions: 1. Q-Sepharose HP, elution with a linear gradient from 10 – 200 mM $MgCl_2$ in 10 mM Tris-HCl, pH 7.8. 2. MonoQ, elution with a linear gradient from 10 – 100 mM $NaP0_4$ buffer, pH 7.8. 3. Gel filtration on Superdex 200. In each case, the location of the recombinant protein was monitored by analysis of fractions emerging from the columns by SDS-PAGE. The final product, which was >98% pure as judged by Coomassie staining, was used to immunize rabbits and for affinity purification of the resulting antibody as described (Tian et al. 2006).

An antibody to human TBCD was raised in rabbits (Sigma-Genosys Inc., Woodlands, TX) using a hapten-conjugated peptide (AGGPEEEAEDETLA) corresponding to amino acids 9-22 of the protein sequence. A full-length cDNA encoding human TBCD was inserted into the pET23d vector, checked by DNA sequencing, and the recombinant protein expressed in *E. coli* BL21(DE3) cells following induction with IPTG. All the recombinant protein was found to be completely insoluble. A total extract of inclusion bodies containing the recombinant protein was resolved by 7% SDS-PAGE. The band corresponding to the recombinant protein, which migrated with an apparent molecular mass of 120 kDa, was used to affinity purify the human anti-TBCD antibody as described (Tian et al. 2006).

Cell Culture, Transfection and Immunofluorescence

Human (HeLa, U2OS, HEK293, T98G) cells were grown in DMEM supplemented with 10% fetal bovine serum. Bovine (EBTr, MDBK) cells were grown in MEM supplemented with 10% fetal bovineserum. All cells were seeded onto glass coverslips and allowed to

grow to a density corresponding to about 30% confluence. For transfection experiments, cells were transfected using either the FuGENE6 or FuGENE-HD reagent (Roche Diagnostics Inc., Indianapolis, IN) following the procedures recommended by the manufacturer. For adenovirus infection experiments, cells were infected at an approximate MOI of 20. After 20 h (in the case of adenovirus infection) or 36 h (in the case of transfection experiments), cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and stained with an anti-TBCD antibody (to detect the transgene), an anti-α-tubulin antibody (Sigma-Aldrich, St.Louis, MO) (to detect the overall microtubule network), and (in some cases) an anti-His antibody (Bethyl Labs, Montgomery, TX) for the detection of tagged Arl2.

SiRNA Experiments

Oligonucleotides optimized for the siRNA-induced silencing of human Arl2 were purchased from Ambion, Inc. (Austin, TX). These oligonucleotides were used to transfect HeLa cells using the oligofectamine transfection reagent (Invitrogen Inc., Carlsbad, CA) over a concentration range of 10 -100 nmolar. Transfection efficiency was assessed using the fluorescently labeled RISC-Free non-targeting control siRNA oligonucleote (Dharmacon, Inc., Lafayette, CO) and found to be close to 100%. As a control for specificity, we used an siRNA oligonucleotide optimized for silencing a related member of the Arl gene family, Arl3 (see Bhamidipati et al. 2000), as well as mock transfected HeLa cells. Two successive rounds of transfection were done with an interval of 48 h. 8 h after the second Arl2 transfection, the cells were transfected with a construct engineered for the expression of human TBCD. For analysis of the extent of Arl2 depletion, soluble extracts of siRNA or mock transfected cells were prepared 92 h after the initial transfection event by Dounce homogenization in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), followed by centrifugation at 16,000 x g. Aliquots of supernatants containing equal amounts of protein were analyzed by Western blotting using an affinity purified rabbit anti-Arl2 antibody. A mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO) was used as a loading control. For analysis of the effect of human TBCD on the microtubule cytoskeleton in cells depleted of Arl2, cells were fixed and examined by immunofluorescence as described above.

RESULTS AND DISCUSSION

Expression of Bovine and Human TBCD in Human Cells Generates Different Molecular Entities

Amongst mammalian species, TBCD is relatively well conserved: there is 81.4% amino acid sequence identity between bovine and human species (Fig. S1). We were therefore surprised to discover that overexpression of human (rather than bovine) TBCD in HeLa cells has no detectable effect on tubulin or microtubule integrity (Cunningham and Kahn 2008), althougha recent report describes a modest effect of prolonged overexpression of human TBCD on HeLa cell microtubules *in vivo* (Fanarraga et al. 2010). To explore this issue, we embarked on the production of bovine and human TBCD as recombinant proteins in order to examine their biochemical and biological properties in detail. We found that TBCD from either species was expressed in host *E. coli* cells as insoluble inclusion bodies, and that these could not be refolded following denaturation to yield biologically active protein. Moreover, while expression of mammalian TBCD in insect *Sf9* cells led to the generation of a modest (<1% of total protein) amount of soluble recombinant protein, we were unable to purify this material from cell extracts because of massive losses at each chromatographic dimension (data not shown). The reason for this finding is unclear; it could reflect protein instability as a result of the absence of one or more kinds of post-translational modification that are essential for TBCD stability but that do not occur in host insect cells. In any event, we

proceeded to express both bovine and human TBCD in mammalian host cells via the use of adenovirus vectors. We chose to express these proteins in untagged form, so as to avoid potential artifacts arising from modification at either the N- or Ctermini.

The results of these experiments are shown in Fig. 2. Although the level of expression of the recombinant proteins was relatively modest (Fig. 2A), we were able to obtain sufficient quantities to purify both the bovine and human proteins to homogeneity using the same chromatographic dimensions we originally developed for purification of authentic bovine TBCD (Tian et al. 1996). Following purification, recombinant bovine TBCD was obtained as a symmetrical peak upon gel filtration, with an apparent mass of about 170 kDa. Analysis of the purified protein by SDS-PAGE revealed that this material contained equimolar amounts of two molecular species: TBCD and β-tubulin (Fig. 2B and 2C, lane 1). We were unable to find conditions that resulted in the separation of these two species so as to generate native TBCD. We conclude that expression of bovine TBCD in human host cells results in tubulin destruction akin to that observed in transfection experiments in HeLa cells (Bhamidipati et al. 2000), with the resulting formation of a stable TBCD/β-tubulin co-complex (Tian et al. 1996,1997). This purified complex was only very weakly active in CCT-driven tubulin heterodimer assembly assays (data not shown).

In contrast to bovine TBCD, purification of the human homolog yielded a single molecular species of 120 kDa (Fig. 2B, lane 2). This material contained no detectable trace of β-tubulin either by Coomassie staining or as determined by Western blotting with an anti-β-tubulin antibody (Fig. 2C, lane 2). These data are consistent with the reported failure of human TBCD to destroy tubulin and microtubules upon overexpression via transfection in HeLa cells (Cunningham and Kahn 2008).

The unexpectedly different properties of human and bovine TBCD as revealed by their different effects on microtubule integrity upon overexpression could be explained as a species difference. Indeed, it has been reported that deletion of the 15 C-terminal amino acids of bovine TBCD inhibits its ability to disrupt microtubule integrity *in vivo* (Shultz et al. 2008), and there exists a difference between the bovine and human proteins in 4out of the 5 C-terminal residues (Fig. S1). However, the bovine C-terminal 5 residues are not sufficient to confer microtubule disruption activity when substituted into the human sequence (Cunningham and Kahn 2008). In any case, little if any difference in factors that modulate overall microtubule behavior would be expected to exist between bovine and human (or indeed any mammalian) species. In view of these considerations, we undertook a comprehensive analysis of the properties of bovine and human TBCD via biochemical experiments *in vitro* as well as biological experiments via their expression in cultured cells.

Biochemical Properties of Recombinant Human TBCD

We assessed the properties of our recombinant human TBCD in several ways. First, we compared its activity with that of authentic (i.e. non-recombinant) bovine TBCD in *in vitro* folding reactions containing purified CCT and TBCD alone (so as to generate β-tubulin/TBCD co-complexes). Second, we measured the activity of authentic bovine and recombinant human TBCD in back reactions in which we compared their ability to disrupt native tubulin heterodimers, looking for the capture of dislocated β-tubulin as a β-tubulin/TBCD co-complex. In both these experiments, we found that the activity of human recombinant TBCD was indistinguishable from its authentic bovine counterpart (Fig. 3A,B). Finally, we compared the activity of authentic bovine and recombinant human TBCD in tubulin GAP reactions (Tian et al. 1999). Once again, we found no detectable difference between the activities of authentic bovine and human recombinant TBCD (Fig. 3C). We conclude that recombinant human TBCD functions as efficiently as the bovine protein

purified from testis tissue in *in vitro* CCT-mediated folding reactions and tubulin disassembly reactions, as well as in tubulin GAP reactions.

Effect of Bovine and Human TBCD on Tubulin and Microtubule Integrity *In Vivo*

Because we found no difference in the ability of bovine tissue-derived TBCD and recombinant human TBCD to participate in *in vitro* tubulin folding or GAP reactions, the failure of human TBCD to destroy tubulin and microtubules upon overexpression in cultured cells is puzzling. To gain insight into this phenomenon, we expressed the bovine and human proteins in cells of bovine (EBTr) and human (HeLa) origin. In each case, cells expressing either bovine or human TBCD were examined by immunofluorescence microscopy using anti-TBCD and anti-α-tubulin antisera, the latter to assess effects on the microtubule cytoskeleton. We found that overexpression of bovine TBCD resulted in the thinning or obliteration of microtubules in both bovine and human cells (Fig. 4A,C). On the other hand, parallel experiments done with human TBCD had no observable effect on microtubules in HeLa cells, but did result in either the destruction or conspicuous thinning of microtubule density in EBTr cells (Fig. 4B,D). These phenotypes were equally evident when overexpression was driven by infection with recombinant adenoviruses (Fig. S2) rather than transfection: this is in spite of the fact that there is evidence that adenovirus infection induces a stabilizing effect on host cell microtubule dynamics (Warren et al. 2006). We extended our experiments to include the effect of overexpression of human TBCD in additional cell lines of human and bovine origin, and found that the results paralleled those obtained for HeLa and EBTr cells: overexpression of bovine TBCD resulted in microtubule destruction in all cases, while human TBCD had no observable effect (Fig. S3). We conclude that the phenotypic difference resulting from overexpression of bovine and human TBCD is ascribable to the species of origin rather than to different cell types.

Given that human and bovine TBCD are indistinguishable as measured by their biochemical properties in all tubulin folding, tubulin destruction and tubulin GAP reactions done *in vitro* (Fig. 3), these data imply that the different effect on microtubule behavior that results from expression of bovine and human TBCD *in vivo* might be caused by one or more effectors that influence the activity of TBCD. An obvious candidate for such an effector is Arl2, a small GTPase belonging to the Ras superfamily that protects tubulin and microtubules from destruction when co-expressed in HeLa cells overexpressing bovine TBCD (Bhamidipati et al. 2000). Indeed, we found that in all cases where expression of TBCD affected the microtubule cytoskeleton, co-expression of Arl2 protected cells from TBCD-mediated changes in microtubule phenotype (Fig. 4E,F,G).

Depletion of Arl2 Results in Human TBCD-mediated Microtubule Disruption In Vivo

Arl2 sequences are very highly conserved amongst mammalian species: within the 184 amino acid polypeptide, only three amino acid substitutions (at positions 108, 141 and 182) distinguish the human and bovine homologs (Fig. S4). We therefore considered the possibility that the greater propensity of bovine TBCD to disrupt microtubule integrity *in vivo* (Fig. 4A,B) might reflect differences in the ability of Arl2 to interact with bovine and human TBCD. To explore this issue, we determined the effect of Arl2 depletion via siRNA on the ability of overexpressed human TBCD to interfere with microtubule integrity in HeLa cells. We first analyzed the effect on Arl2 levels of siRNA treatment using two oligonucleotides optimized for targeting human Arl2 mRNA. Western blotting of extracts of HeLa cells treated with these siRNAs for 92 h showed a relative depletion of Arl2 (compared to controls) of at least 75% (Fig. 5A). We then examined the effect of overexpression of human TBCD on Arl2 siRNA-treated HeLa cells. We found that, in contrast to controls (i.e. mock transfected HeLa cells or HeLa cells transfected with siRNA targeting Arl3, a different member of the Arl family of proteins), overexpression of human

TBCD in Arl2 siRNA-treated cells resulted in a conspicuous thinning of microtubules (Fig. 5B,C). In addition, the surviving microtubules in these cells tended to be organized in a circumferential fashion, consistent with the reported dissociation of microtubules from the centrosome as a consequence of overexpression of TBCD (Fanarraga et al. 2010). We conclude that, like its bovine homolog, human TBCD is capable of inducing tubulin and microtubule destruction *in vivo*, but that this reaction is inhibited by interaction of TBCD with Arl2 and potentially other interacting effectors.

Effect of Recombinant Arl2 on Tubulin Heterodimer Assembly *In Vitro*

We considered the possibility that the extended delay or failure of human TBCD to destroy microtubules upon overexpression in HeLa cells might be attributed to a protective effect conferred by a higher level of expression of Arl2. However, analysis of total extracts of bovine (EBTr) and human (HeLa) cells by Western blotting with our anti-Arl2 antibody (data not shown) suggested little significant difference in the level of expression of Arl2 in these cell lines. The difference in the efficiency with which bovine and human TBCD influence microtubule integrity *in vivo* might therefore be ascribable to a difference in affinity of the two proteins either for their target (the tubulin heterodimer) or their regulatory effector (Arl2) or both. To address this issue, we tested the effect of including recombinant Arl2 in *in vitro* folding assays containing TBCD. Although we found that human Arl2 is expressed in host *E. coli* cells as a relatively abundant soluble protein that could be readily purified to homogeneity, we found that this material failed to form a stable complex with TBCD (data not shown). The explanation for this unexpected observation may lie in the fact that small G proteins (including those of the Arl family) depend upon guanine nucleotide exchange factors (GEFs) in order to transition between GDP and GTP bound forms (reviewed in Bos et al., 2007). In addition, they are post-translationally modified at their N-termini with myristoyl, prenyl or acetyl groups (Graham, 2004). Thus, we found that expression of human Arl2 via *in vitro* transcription/translation in a eukaryotic cell-free extract led to the generation of two molecular species each with a distinct mobility upon native gel electrophoresis. On the other hand, the corresponding recombinant protein migrated under identical conditions as closely resolved major and minor bands, with the major band corresponding in mobility to the slower migrating band produced by transcription/translation (Fig. S5). Moreover, the observation that Arl2 is found to be associated with both TBCD and PP2A in cell extracts (Shern et al. 2003) suggests that the Arl2/TBCD interaction may be indirect.

TBCD/β-tubulin Co-complexes Are Formed at Reduced Levels of Expression of Bovine TBCD *In Vivo*

In the absence of a straightforward means of making functional Arl2 suitable for *in vitro* biochemical assays, we explored the ability of bovine TBCD to disrupt the tubulin heterodimer at a relatively low level of expression in HeLa cells, reasoning that under these conditions, at least some of the bovine protein might be produced as a monomeric species rather than exclusively as a co-complex with β-tubulin. However, this experiment showed that even under conditions where bovine TBCD was expressed with an overall abundance of less than about 0.4% of total protein, the recombinant protein was always found in association with at least some β-tubulin (Fig. S6). This experiment reinforces our conclusion that bovine TBCD has a greater propensity to disrupt the tubulin heterodimer upon overexpression *in vivo* compared to its human homolog.

Analysis of Authentic and Recombinant Bovine TBCD by Mass Spectrometry

We also compared authentic and recombinant bovine TBCD by mass spectrometry, looking for evidence of one or more post-translational differences conferred by the human host cells that might explain the enhanced ability of this protein to destroy tubulin *in vivo*. These

experiments did not reveal any difference between the masses of peptides derived from the authentic and recombinant bovine TBCD proteins, with a coverage of about 59% (data not shown). Thus, we consider a difference in posttranslational modification between authentic bovine TBCD and its recombinant counterpart made via expression in human cells to be unlikely. However, the possibility that human cells modify bovine TBCD in such a way as to enhance its ability to destroy tubulin heterodimers cannot be formally ruled out.

Conclusions

TBCD is a protein central to the pathway leading to the *de novo* assembly of the tubulin heterodimer. Since tubulin biogenesis is an essential process common to all eukaryotic species, it is puzzling that bovine TBCD disrupts tubulin and microtubule integrity upon overexpression (the heterodimer assembly reaction driven in reverse) (Bhamidipati et al. 2000), while the human protein does not (Cunningham and Kahn 2008), or at least does so with a significantly reduced efficiency and on an extended time-scale (Fanarraga et al. 2010). Consistent with the expected evolutionary conservation of the tubulin heterodimer assembly pathway, our experiments show that TBCD from two different mammalian species share identical biochemical properties with respect to tubulin heterodimer assembly *in vitro*. Taken together, our data favor the view that the relatively low propensity of human TBCD to destroy tubulin and microtubules *in vivo* is because of an enhanced protective effect conferred by a higher affinity of Arl2 (and potentially other interacting effectors) for human TBCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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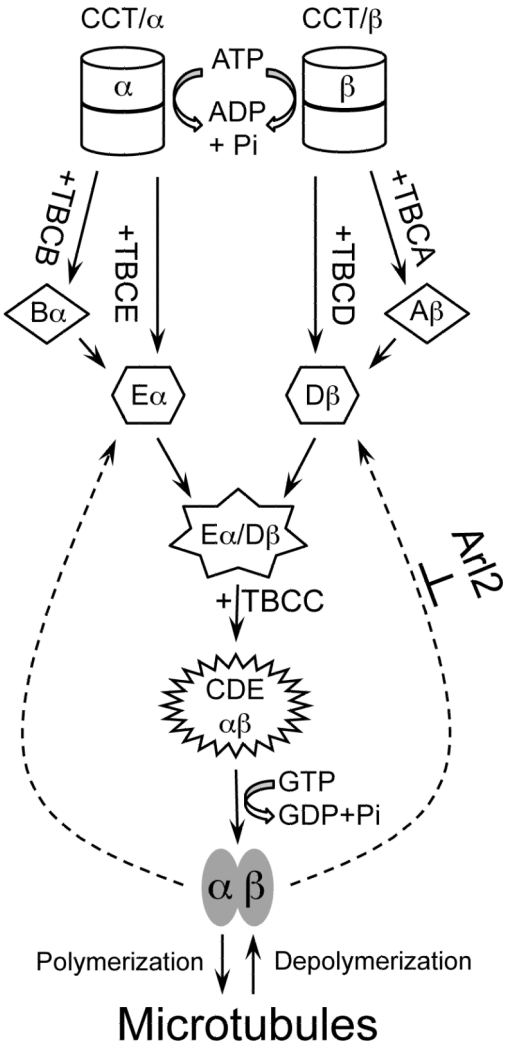

Figure 1. The Tubulin Heterodimer Assembly Pathway

The α/β-tubulin heterodimer is assembled as a result of the action of a series of tubulin-specific chaperones (TBCA-E) whose function is to assemble the tubulin heterodimer. Nascent α- and β-tubulin polypeptides are protected from off-pathway folding pathways by interaction with the cytosolic chaperonin, CCT. The chaperonin provides a sequestered chamber within which productive folding can take place in the absence of interactions with other molecules that might lead to misfolding. One or more cycles of ATP-dependent interaction with CCT leads to the production of quasi-native tubulin folding intermediates, defined by the incorporation of GTP. These intermediates are captured upon discharge from CCT either by TBCA (in the case of β-tubulin) or TBCB (in the case of α-tubulin). TBCA/β-tubulin (Aβ) and TBCB/α-tubulin (Bα) transfer their respective target proteins to TBCD and TBCE. These two complexes (i.e. TBCD/β-tubulin [Dβ] and TBCE/α-tubulin [Eα]) converge to form a supercomplex (TBCD/β/TBCE/α). Entry of TBCC into this supercomplex triggers the hydrolysis of GTP in the β-tubulin E-site. This reaction acts as a switch for the release of the newly formed α/β-tubulin heterodimer, which is then competent for GTP exchange and polymerization into microtubules. The folding reaction is reversible: TBCD and TBCE are capable of interacting with the native heterodimer, sequestering the β- and α-subunit, respectively (the so-called backreaction, shown as dashed lines in the Figure). Arl2 interacts with TBCD, sequestering it and thereby preventing it from disrupting the

native tubulin heterodimer. Based on Tian et al. 1997, Bhamidipati et al. 2000 and Cowan and Lewis, 2002.

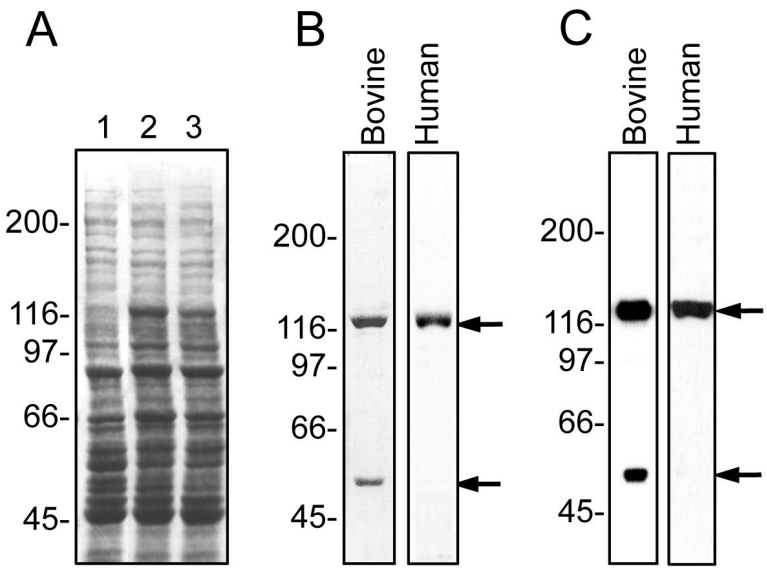

Figure 2. Purification of Recombinant Bovine and Human TBCD

Bovine and human TBCD were expressed independently in HeLa cells via infection with recombinant adenoviruses and purified by FPLC using various chromatographic dimensions (see Methods). A) Analysis by SDS-PAGE of equal amounts of protein contained in unfractionated cells extracts. Lane 1, uninfected cells; lanes 2 and 3, cells infected with recombinant adenoviruses engineered for the expression of bovine and human TBCD, respectively. B) Purified proteins detected by staining with Coomassie blue. C) Western blot of the materials shown in B) with antibodies to TBCD and β-tubulin. Note the detection of β-tubulin in the case of bovine TBCD (lane 1) but not human TBCD (lane 2). Molecular mass standards are shown at the left. Upper and lower arrows in (B) and (C) mark the migration positions of TBCD and β-tubulin, respectively.

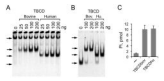

Figure 3. Comparison of Tissue-derived and Recombinant TBCD's in *In Vitro* CCT-mediated Reactions, Tubulin Destruction Reactions and Tubulin GAP Reactions

A) *In vitro* folding reactions were performed as described (Tian et al. 1997) by presentation of ^{35}S-labeled, urea-unfolded β-tubulin target protein to 1.0 μg of purified CCT in the presence of ATP, GTP and either tissue-derived bovine (Dbov) or recombinant human TBCD (Dhu) in the amounts shown in the Figure. B) Approximately 100 ng of native tubulin heterodimers ^{35}S-methionine-labeled in their β-subunits by translation *in vitro* were purified (lane 1) and incubated with either 100 ng or 200 ng of either bovine TBCD (purified from testis tissue) (lanes 2 and 3) or with recombinant human TBCD (lane 4 and 5). Reaction products were resolved on 4.5% polyacrylamide gels. Arrows in A and B (top to bottom) denote the migration positions of the CCT/β-tubulin binary complex, the bovine TBCD/β-tubulin co-complex, the human TBCD/β-tubulin co-complex, and native α/β tubulin heterodimers. C) GAP activity in reactions containing γ-^{23}P-labeled GTP, unlabeled bovine brain tubulin, TBCC, and either no TBCD (as a negative control, lane 1) or equimolar amounts of bovine TBCD purified from testis tissue (lane 2) or human recombinant TBCD (lane 3). Error bars represent standard deviations based on 3 independent experiments.

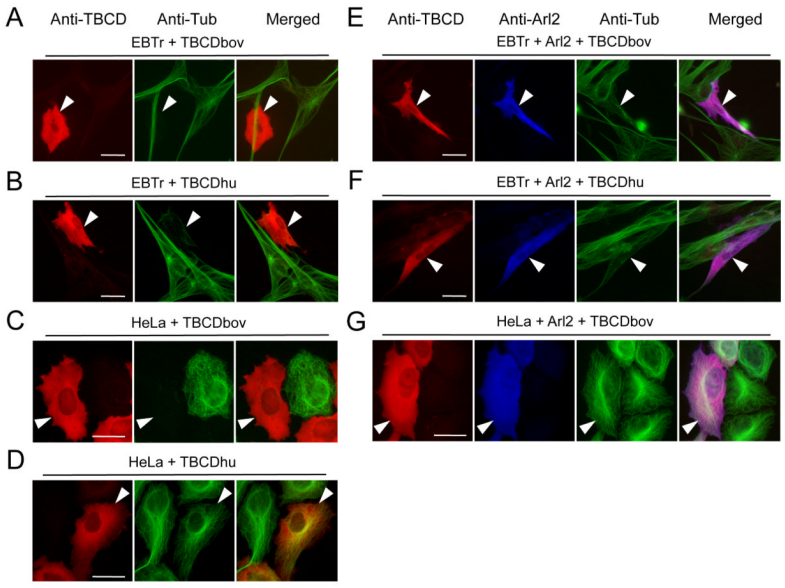

Figure 4. Overexpression of Bovine and Human TBCD in Human and Bovine Cells: Effect on Microtubule Integrity and Rescue by Co-expression of Arl2

Sequences engineered for the expression of either bovine (A,C,E,G) or human (B,D,F) TBCD were introduced into bovine (EBTr) (A,B,E,F) or human (HeLa) cells (C,D,G) by transfection. In some cases (E-G) the cells were co-transfected with sequences engineered for the expression of HA-tagged human Arl2. 36 h post transfection, the cells were fixed and examined by indirect immunofluorescence using rabbit anti-TBCD antisera (to detect the transgene: shown in red), a monoclonal anti-α-tubulin antibody (to detect the microtubule network: shown in green) and an anti-His polyclonal antibody raised in chicken (to detect Arl2, shown in blue). Cells expressing the transgene are arrowed. Note a) the thinning or obliteration of microtubules as a result of overexpression of bovine TBCD in bovine (EBTr) (A) and human (HeLa) cells (C); b) the thinning of microtubules as a result of overexpression of human TBCD in bovine (EBTr) (B) but not in HeLa cells (D); and c) the rescue of TBCD-mediated microtubule thinning or destruction as a result of coexpression of Arl2 in any context where this occurs (E-G). Bar = 10 μm.

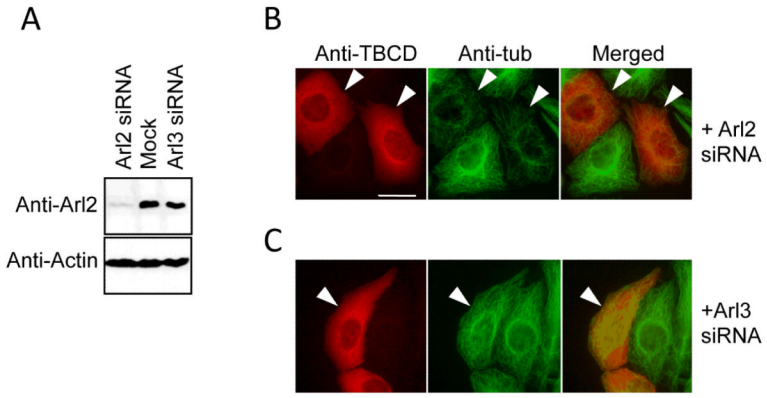

Figure 5. Overexpression of Human TBCD Results in Microtubule Destruction in Arl2-Depleted HeLa Cells

(A) Συππρεσσιον Οφ Αρλ2 εξπρεσσιον ω¯ια σιPNA. Ωεστερν βλοτ οφ εξτραχτσ οφ ΗεΛα χελλσ τρεατεδ ωιτη σιPNAσ ταργετινγ Αρλ2 (λανε 1), μοχκ–τρανσφεχτεδ ΗεΛα χελλσ (λανε 2), ορ ΗεΛα χελλσ τρεατεδ ωιτη σιPNAσ ταργετινγ Αρλ3, α μεμ βερ Οφ τηε Αρλ φαμιλψ τηατ ισ διστινχτ φρΟμ Αρλ2 (λανε 3). ΝΟτε α δεπλετιΟν οφ Αρλ2 ρελατιω¯ε τΟ τηε χΟντρΟλσ σηΟων ιν λανεσ 2 ανδ 3 οφ ατ λεαστ 75%. ΛΟα δινγ χΟντρΟλσ δΟνε ωιτη αν αντι–β-actin antibody are shown below. B, C) HeLa cells overexpressing human TBCD (shown in red) and depleted of either Arl2 (B) or Arl3 (C). Microtubules were detected using a monoclonal anti-α-tubulin antibody (shown in green). Note the conspicuous thinning of microtubules in cells expressing human TBCD and depleted of Arl2 (arrowed in (B)). Bar = 10 μm.

Table 1

Purification of Recombinant TBCD from Adenovirus-infected HeLa Cells

Chromatographic Dimension	Anion Exchange (QHP)	Hydroxylapatite (Pentax)	Anion Exchange (MonoQ)	Gel Filtration (Superose 6)
% Purity [+]	5	15	50	>95
Fold Enrichment [+ *]	3	9	30	60

[+] Values are approximate

[*] Relative to the initial soluble extract used as starting material (see Methods)