A Pachygyria-causing α -Tubulin Mutation Results in Inefficient Cycling with CCT and a Deficient Interaction with TBCB

Guoling Tian,* Xiang-Peng Kong,* Xavier H. Jaglin,[†] Jamel Chelly,[†] David Keays,[‡] and Nicholas J. Cowan*

*Department of Biochemistry, New York University Medical Center, New York, NY 10016; [†]Institut Cochin, Université René Descartes, Paris F-75014, France; and [‡]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, United Kingdom

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The agyria (lissencephaly)/pachygyria phenotypes are catastrophic developmental diseases characterized by abnormal folds on the surface of the brain and disorganized cortical layering. In addition to mutations in at least four genes—*LIS1*, *DCX*, *ARX* and *RELN*—mutations in a human α -tubulin gene, *TUBA1A*, have recently been identified that cause these diseases. Here, we show that one such mutation, R264C, leads to a diminished capacity of de novo tubulin heterodimer formation. We identify the mechanisms that contribute to this defect. First, there is a reduced efficiency whereby quasistative α -tubulin folding intermediates are generated via ATP-dependent interaction with the cytosolic chaperonin CCT. Second, there is a failure of CCT-generated folding intermediates to stably interact with TBCB, one of the five tubulin chaperones (TBCA–E) that participate in the pathway leading to the de novo assembly of the tubulin heterodimer. We describe the behavior of the R264C mutation in terms of its effect on the structural integrity of α -tubulin and its interaction with TBCB. In spite of its compromised folding efficiency, R264C molecules that do productively assemble into heterodimers are capable of copolymerizing into dynamic microtubules in vivo. The diminished production of *TUBA1A* tubulin in R264C individuals is consistent with haploinsufficiency as a cause of the disease phenotype.

INTRODUCTION

Proper brain function depends on the correct positioning of neurons as a result of directed migration during development (Ayala *et al.*, 2007). Neuronal migration disorders are a group of brain developmental diseases characterized by neuronal migration defects that result in severe cortical malformations. In its most severe form (lissencephaly), the surface of the brain has a smooth appearance, four abnormal cortical layers, and a variably hypoplastic cerebellum (Dobyns and Truwit, 1995; Dobyns *et al.*, 1996). In a less severe form (pachygyria), the disease is characterized by a simplified pattern of folds on the surface of the brain. In either event, a consequence of impaired neuronal migration is not only aberrant lamination in the brain, but ultimately behavioral deficits: humans with lissencephaly/pachygyria are mentally retarded and frequently suffer from epilepsy (Francis *et al.*, 2006). The disease involves mutations in a number of genes.

In classical lissencephaly, the cause is ascribable to sporadic mutations in the *LIS1* gene, resulting in haploinsufficiency. Lis1 is a ubiquitously expressed 45-kDa protein; it plays multiple functional roles that include interactions with microtubules via cytoplasmic dynein. Lis1 binds to dynein

via several distinct sites (Faulkner *et al.*, 2000; Smith *et al.*, 2000; Tai *et al.*, 2002) and to several other proteins (e.g., mNudC, mNudE, and NudEL) originally identified in *Aspergillus* as being involved in nuclear distribution (Efimov and Morris, 2000; Kitagawa *et al.*, 2000), thus linking Lis1 with nucleokinesis. Lis1 also binds to catalytic dimers of the brain cytosolic platelet activating factor acetylhydrolase, which in turn enhances *N*-methyl-D-aspartate receptor currents (Tabuchi *et al.*, 1997). Importantly, suppression of Lis1 via small interfering RNA causes an accumulation of multipolar progenitor cells within the subventricular zone of embryonic rat brains. There is also an abolition of interkinetic nuclear oscillations of the radial progenitors in the ventricular zone and an accompanying inhibition of cell division (Hatten, 2005; Tsai *et al.*, 2005).

In its X-linked form, lissencephaly is caused by both familial and sporadic mutations in the gene (*DCX*) encoding the microtubule associated protein doublecortin (Gleeson *et al.*, 1998). It has been demonstrated that doublecortin coassembles with brain tubulin, stimulating polymerization into microtubules that contain 13 protofilaments (Francis *et al.*, 1999). *DCX* associates with the dynein complex, an interaction that might be mediated by the previously identified interaction between *DCX* and Lis1 (Tanaka *et al.*, 2004). Mutations that cause lissencephaly are clustered in the two tubulin binding domains of *DCX*, and their presence impairs polymerization of microtubules both in vitro and in vivo (Sapir *et al.*, 2000), thus implicating correct binding of *DCX* to microtubules as critical for proper neuronal migration. *DCX* does not bind to the tubulin heterodimer, but it does nucleate microtubule growth and acts as a potent anticatas-

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Address correspondence to: Nicholas J. Cowan (cowann01@med.nyu.edu).

trophe factor that stabilizes microtubules by linking adjacent protofilaments in the microtubule lattice. The enrichment of DCX at the distal ends of neuronal processes has led to the suggestion that the function of DCX in neurons is to drive the assembly and stabilization of noncentrosomal microtubules in DCX-enriched zones (Moore et al., 2004, 2006). Surprisingly, knockout of *Dcx* in mice does not result in defects in cortical neuronal lamination or positioning (Corbo et al., 2002). However, suppression of DCX expression reveals that the protein is required for radial migration in rat neocortex (Bai et al., 2003), and the absence of DCX does result in a severe morphological defect that implicates its function in the maintenance of bipolar shape and nuclear translocation during migration in the adult forebrain (Kozumi et al., 2006). DCX may also function in roles that are not directly related to microtubules. For example, DCX interacts with c-Jun NH₂-terminal kinase (JNK) and JNK interacting protein, implicating the involvement of DCX in a signaling pathway (Gdalyahu et al., 2004).

Perturbations of the laminar architecture of the cortex also result from mutations in the Aristaless-related homeobox gene *ARX* (Strømme et al., 2002), the extracellular matrix protein *reelin* (Hong et al., 2000), and the very-low-density lipoprotein receptor (*VLDLR*) (Boycott et al., 2005). *ARX* has been identified as the gene associated with an X-linked human brain malformation, and with abnormal genitalia (Kitamura et al., 2002). *Reelin* was first implicated in neuronal migration by studying the naturally occurring mouse mutant *reeler*, which displays an inversion of the normal inside-out order of cortical neurons (D'Arcangelo et al., 1995; Trommsdorff et al., 1999). Together, mutations in *DCX*, *LIS1*, *ARX*, *reelin*, and *VLDLR* account for <50% of the cases of the lissencephaly/pachygyria spectrum of phenotypes (Francis et al., 2006). Very recently, mutations in the α -tubulin gene *TUBA1A* have also been shown to cause neuronal migration disorders (Keays et al., 2007; Poirier et al., 2007). These observations reinforce the notion that a common feature of many forms of lissencephaly/pachygyria is the disruption of key elements of microtubule behavior. In mutations involving α -tubulin, this might occur either via defects in the tubulin heterodimer assembly pathway or via a mechanism in which proper interactions between α -tubulin and critical effectors of microtubule function are compromised. Here, we describe the elucidation of the mechanisms whereby a recurrent pachygyria-causing mutation, R264C, in *TUBA1A* results in a compromised efficiency of de novo α/β -tubulin heterodimer formation.

MATERIALS AND METHODS

Cloning and In Vitro Translation

A full-length cDNA encoding the human *TUBA1A* sequence was generated by polymerase chain reaction (PCR) by using a human brain cDNA library (Clontech, Mountain View, CA) as template. The PCR product was cloned into the pcDNA 3.1+ vector (Invitrogen, Carlsbad, CA) and checked by DNA sequencing. The R264C mutation was introduced by site-directed mutagenesis by using a QuikChange II kit (Stratagene, La Jolla, CA). A tag encoding the FLAG epitope (DYKDDDDK) was incorporated into the *TUBA1A* wild-type or R264C sequence by PCR in such a way that it substituted either for the C-terminal tyrosine residue or for the 11 C-terminal amino acids of the α -tubulin sequence. These products were also cloned into the pcDNA3.1+ vector. All constructs were checked by DNA sequencing. Transcription/translation reactions were done at 30°C for 60 min in 25 μ l of rabbit reticulocyte lysate (TNT; Promega, Madison, WI) containing [³⁵S]methionine (specific activity, 1000 Ci/ μ mol; 10 μ Ci/ μ l). In kinetic experiments, aliquots (1.5 μ l) were withdrawn from the reaction at various times, diluted into 5 μ l of gel loading buffer (gel running buffer supplemented with 10% glycerol and 0.1% bromophenol blue), and stored on ice before resolution on a nondenaturing gel (Tian et al., 1997). For the generation of α -tubulin-labeled heterodimers, transcription/translation reactions were chased for a further 15 min after the

addition of native bovine brain tubulin to 0.2 mg/ml. In some experiments, the labeled product was isolated on microcolumns of DEAE Sephacel as described previously (Tian et al., 1997). Labeled reaction products were detected by autoradiography after resolution on either SDS-polyacrylamide gel electrophoresis (PAGE) or on native polyacrylamide gels as described previously (Tian et al., 1997).

Sensitivity to Proteolytic Digestion

Tubulin heterodimers containing [³⁵S]methionine-labeled wild-type or R264C mutant *TUBA1A* sequences were generated by in vitro transcription/translation and purified as described above. The reaction products were subjected to digestion with proteinase K, and the reaction products were analyzed by SDS-PAGE as described previously (Tian et al., 2006).

In Vitro Folding Reactions

In vitro folding assays were done in folding buffer containing CCT, ATP, guanosine triphosphate (GTP), tubulin chaperones (TBCB, TBCC, TBCD, and TBCE), and purified native tubulin heterodimer as described previously (Tian et al., 1995b; Tian et al., 1996). Target proteins (i.e., wild type or R264C *TUBA1A* α -tubulin) were expressed as either unlabeled or ³⁵S-labeled proteins in *Escherichia coli*, and the inclusion bodies were purified and unfolded in 8 M urea as described previously (Gao et al., 1992). Reaction products were analyzed either by electrophoresis on native polyacrylamide gels as described previously (Zabala and Cowan, 1992), or on a 2.2-ml gel filtration column of Superose 6 equilibrated and run on a Smart system (GE Healthcare, Piscataway, NJ) in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EGTA. Back reactions (see text) were done by incubating purified wild-type or R264C tubulin heterodimers [³⁵S]methionine-labeled by in vitro transcription/translation in their α -subunit with a twofold molar excess of TBCD and a fourfold stoichiometric excess of TBCB for 30 min at 30°C as described previously (Tian et al., 1997). In some experiments, folding reactions were done with unlabeled target proteins in reactions containing CCT, ATP, and 20 μ M [α -³²P]GTP (specific activity, 100 Ci/mmol) (Tian et al., 1995a). In kinetic experiments done with [α -³²P]GTP, reactions were terminated at various time intervals by withdrawing an aliquot from the reaction mixture and quenching further formation of labeled I_Q intermediates by the addition of unlabeled GTP to a final concentration of 2 mM. Samples were stored on ice before analysis on nondenaturing gels. In experiments to determine the stability of I_Q intermediates, folding reactions done for 1 h in the presence of [α -³²P]GTP were quenched with unlabeled GTP and the incubation continued at 30°C for various time intervals before analysis by native PAGE.

Microtubule Copolymerization Experiments

Products of in vitro translation reactions or CCT-mediated in vitro folding reactions were mixed with depolymerized bovine brain microtubules, and they were taken through successive cycles of polymerization and depolymerization as described previously (Tian et al., 1997). At the end of each of these cycles, aliquots containing equal amounts of depolymerized material were removed and analyzed by either native gel electrophoresis or by scintillation counting.

Cell Culture, Transfections, and Immunofluorescence

Constructs were transfected into HeLa cells grown on glass coverslips in DMEM containing 10% fetal calf serum by using the FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Thirty-six hours posttransfection, cells were fixed with paraformaldehyde. In regrowth experiments to determine the dynamic behavior of microtubules, cells were incubated for 2 h with 10 μ M nocodazole 36 h posttransfection, restored to drug-free medium, and fixed at various brief intervals thereafter. Cells were stained with a polyclonal anti-FLAG antibody and a monoclonal anti- β -tubulin antibody (Sigma-Aldrich, St. Louis, MO).

Computational Methods

A model of human α -tubulin was built by homology modeling using available structures (Research Collaboratory for Structural Bioinformatics PDB code 1TUB). The image in Figure 5A was rendered using PyMOL (<http://www.pymol.org>).

RESULTS

The R264C α -Tubulin Mutation Results in Inefficient Native Heterodimer Production on Translation In Vitro

In higher eukaryotes, assembly of the α/β -tubulin heterodimer, the subunit from which microtubules polymerize, cannot occur spontaneously. Rather, the process involves interaction with a series of molecular chaperones (Tian et al., 1997; Cowan and Lewis, 2002; Szymanski, 2002). Newly synthesized α - and β -tubulin polypeptides are first captured

in a partly unfolded conformation by the chaperone prefoldin, which delivers them to the ATP-dependent chaperonin CCT (Vainberg *et al.*, 1998). Successive cycles of iterative annealing within the chaperonin cavity result in the generation of folding intermediates that are quasi-native in the sense that they contain a folded GTP-binding pocket (Tian *et al.*, 1995a), but these molecules are still not competent to assemble so as to produce functional heterodimers. For this to occur, a further series of interactions is required with a series of downstream tubulin chaperones termed TBCE-E (Figure 1A).

We first investigated the ability of sequences containing the R264C pachygyria-causing mutation (Keays *et al.*, 2007) to become incorporated into tubulin heterodimers by expressing the mutant protein in rabbit reticulocyte lysate in the presence of [³⁵S]methionine. The reticulocyte-derived cell-free cocktail contains all the components required for de novo tubulin heterodimer assembly (Cleveland *et al.*, 1978). The reaction products were analyzed by electrophoresis under both denaturing (SDS-PAGE) and nondenaturing gel conditions. We found that the R264C mutant was expressed as efficiently as its wild type counterpart as judged by the yield of labeled protein analyzed under denaturing conditions (Figure 1B). However, upon analysis under native conditions, the yield of product that migrated as native heterodimer was reduced by approximately fivefold in the case of the mutant protein (Figure 1C). The reduced level of tubulin heterodimer formation in the case of the R264C mutant was reflected at least in part by an enhanced retention of total label in the CCT/ α -tubulin binary complex (~11% in the wild-type protein vs. ~18% in the R264C mutant). Consistent with this result, kinetic analysis of these reactions showed a slower rate of accumulation of native heterodimer in the case of the R264C mutant (Supplemental Figure 1), suggesting one or more defects in the heterodimer assembly pathway. To confirm that the material produced in reduced yield in these reactions was indeed native, we subjected the wild type and mutant cell-free translation products to two successive cycles of polymerization and depolymerization with added purified bovine brain microtubules. In this experiment, we found that wild-type and mutant heterodimers produced by transcription/translation in rabbit reticulocyte lysate cocycled with brain tubulin with indistinguishable efficiency (Figure 1D). We conclude that although the R264C mutant α -tubulin protein is compromised in terms of its ability to assemble into native tubulin heterodimers in good yield, once assembled, mutant-containing heterodimers can efficiently copolymerize into microtubules in vitro.

R264C Mutant α -Tubulin Acquires Bound GTP at a Reduced Rate and Does Not Stably Interact with TBCB

We first sought to determine whether the R264C mutation resulted in a compromised ability of the α -tubulin target protein to acquire a quasistable state (termed I_Q , defined as containing GTP bound at the N-site; Tian *et al.*, 1995a) as a result of ATP-dependent cycling with CCT. To do this, equal amounts of unlabeled, urea-unfolded wild-type or mutant polypeptides made by expression in *E. coli* were presented by sudden dilution into buffer containing CCT, ATP, and [α -³²P]GTP. After incubation at 30°C, the reaction products were resolved on a nondenaturing gel. The result of this experiment showed that the mutant polypeptide acquired labeled GTP as a result of ATP-dependent cycling with chaperonin much more slowly than its wild-type counterpart (Figure 2, A and C). To see whether the reduced rate of formation of I_Q intermediates of the R264C mutant reflected a relative instability of this species, we measured the half-life

Figure 1. The R264C α -tubulin mutation produces assembly competent heterodimers in reduced yield on transcription/translation in vitro. (A) Chaperone-dependent interactions in the tubulin heterodimer assembly pathway. Newly synthesized α - and β -tubulin polypeptides are delivered to the cytosolic chaperonin, CCT (Gao *et al.*, 1992), via the chaperone prefoldin (PFD) (Vainberg *et al.*, 1998). One or more successive rounds of binding and ATP-dependent release within the CCT cavity results in the generation of quasistable intermediates, termed I_Q in the case of α -tubulin and defined as containing the native (N-site) guanine nucleotide binding pocket (Tian *et al.*, 1995a). These intermediates are captured and stabilized by interaction with TBCs: TBCA and TBCD in β -tubulin and TBCB and TBCE in α -tubulin. The α - and β -tubulin folding pathways converge via interaction between TBCD/ β -tubulin and TBCE/ α -tubulin to form a supercomplex. Entry of TBCE into this supercomplex triggers the hydrolysis of GTP by β -tubulin; this acts as a switch for the release of the heterodimer, which is then competent for nucleotide exchange and polymerization into microtubules (MT) (Tian *et al.*, 1997; Cowan, 2002). In addition to their function in heterodimer assembly, TBCD and TBCE can interact with the native tubulin heterodimer (the back reaction; shown as dashed lines in the Figure), disrupting it and reforming either the TBCD/ β -tubulin or TBCE/ α -tubulin complex (Tian *et al.*, 1997; Bhamidipati *et al.*, 2000). (B and C) Analysis on denaturing (SDS-PAGE) (B) and on nondenaturing (C) gels of the products of transcription/translation reactions done with wild-type TUBA1A and the corresponding R264C mutant. Arrow marks the migration position of α -tubulin in B. Top and bottom arrows in C denote the migration positions of the CCT/ α -tubulin binary complex and native tubulin heterodimers, respectively. (D) Copolymerization of translation products with native bovine brain microtubules. Aliquots taken after successive rounds of polymerization and depolymerization and containing equal amounts of total protein were analyzed by native gel electrophoresis.

of I_Q intermediates in pulse-chase experiments. This experiment showed that wild-type and mutant I_Q intermediates had essentially the same stability (Figure 2, B and D). We conclude that the R264C mutation results in a compromised capacity of CCT to generate quasistable folding intermediates as a result of the chaperonin's ability to efficiently cycle its target protein.

We next tested the ability of CCT-generated α -tubulin I_Q intermediates to interact with the downstream chaperones that participate in the de novo formation of α/β tubulin

Figure 2. Incorporation of GTP into quasinative (I_Q) α -tubulin folding intermediates. (A) Native gel analysis of the products of in vitro folding reactions done in which an equal amount of unlabeled, unfolded wild-type or R264C mutant α -tubulin probe was incubated with CCT in the presence of ATP and α - 32 P-labeled GTP. Aliquots were withdrawn from these reactions at the times shown. (B) Half-life of I_Q intermediates. Wild-type and R264C I_Q intermediates were generated for 1 h as described in A, the reactions were quenched by the addition of unlabeled GTP, and the incubations were continued for the times shown in the figure before their analysis on a native polyacrylamide gel. Arrows in A and B mark the migration position of quasinative CCT-bound α -tubulin (I_Q) folding intermediates. (C and D) Quantitative analysis of experiments as shown in A and B. Triangles and squares show label in wild-type and R264C I_Q intermediates, respectively. Error bars show the SD based on at least three independent experiments.

heterodimers. To do this, [35 S]methionine labeled α -tubulins were expressed in *E. coli* as insoluble inclusion bodies, purified, unfolded in 8 M urea, and diluted into in vitro folding reactions containing CCT, ATP, GTP, and one or more of the chaperones (TBCB-E) required for de novo incorporation of α -tubulin into the heterodimer. After incubation at 30°C, the reaction products were resolved by native PAGE. In these experiments, we noted a conspicuous absence of the TBCB/ α -tubulin intermediate in reactions done with the mutant target protein and TBCB alone. In addition, in fully reconstituted reactions containing TBCC, TBCD, and TBCE (with or without TBCB), the yield of native heterodimers produced in reactions done with the mutant target protein was

reduced by a factor of about five compared with parallel control reactions done with the wild-type protein (Figure 3A). We also analyzed the products of in vitro folding reactions done with CCT and TBCB by gel filtration on Superose 6. In the case of wild type α -tubulin, in addition to the 800-kDa binary complex formed between CCT and the target protein, a labeled product migrating with an apparent molecular mass of $\sim$ 90 kDa was observed. This material has the expected migration characteristics of a complex (TBCB/ α -tubulin) formed between α -tubulin and a single molecule of TBCB. However, in the case of the R264C mutant, no product migrating at 90 kDa was formed (Figure 3B). Together, these data suggested that quasinative mutant α -tubulin polypeptides produced by interaction with CCT are unable to form a stable cocomplex with TBCB.

To reinforce this conclusion, we took advantage of the reversibility of the chaperone-driven tubulin heterodimer assembly reaction, in which native heterodimers can be disrupted by interaction with TBCD. TBCD captures the β -subunit, disrupting the heterodimer (Figure 1A, back-reaction). We prepared native wild-type and R264C mutant-containing heterodimers 35 S-labeled in the α -tubulin subunit by coupled transcription/translation in rabbit reticulocyte lysate. The labeled heterodimers were purified by ion exchange chromatography and incubated with a molar excess of TBCD in the presence of TBCB. Under these conditions, the heterodimer is disrupted by interaction with TBCD such that the β -subunit forms a TBCD/ β -tubulin complex, whereas the uncoupled 35 S-labeled α -subunit is free to form a cocomplex (TBCB/ α -tubulin) with TBCB (Tian *et al.*, 1997; Bhamidipati *et al.*, 2000). Analysis by native PAGE of the products of these reactions showed that, in contrast to wild-type α -tubulin, the labeled heterodimer-derived R264C mutant-containing α -tubulin polypeptide completely failed to yield detectable quantities of the TBCB/ α -tubulin intermediate (Figure 3C). The apparent disappearance of the input label in back reactions done with the R264C α -tubulin mutant presumably reflects either its irreversible denaturation in the absence of a functional interaction with a stabilizing component, or its existence in an unstable complex with TBCB that does not survive native gel electrophoresis. In either event, most of the radioactivity is presumably lost to the reaction vessel walls (Tian *et al.*, 1997), although we note the presence of a slightly enhanced signal at the gel origin that probably represents aggregated material. We conclude that R264C mutant α -tubulin polypeptides generated either as a

Figure 3. The R264C α -tubulin mutation fails to form a stable complex with TBCB. (A) Native gel analysis of the products of in vitro folding reactions done in which [35 S]methionine-labeled, unfolded wild-type or R264C mutant α -tubulin probes were incubated with CCT in the presence of ATP, GTP, and the TBCs shown in the figure. Arrows (top to bottom) show the migration positions of the CCT/ α -tubulin binary complex, the TBCB/ α -tubulin complex, and the native α/β tubulin heterodimer, respectively. (B) Analysis by gel filtration on Superose 6 of the products of in vitro folding reactions done with [35 S]methionine-labeled, unfolded wild-type or R264C mutant α -tubulin probes presented to CCT in the presence of ATP, GTP, and TBCB. Open triangles denote the expected elution positions of the CCT/ α -tubulin and TBCB/ α -tubulin binary complexes; solid arrows show the migration positions of molecular mass markers (670, 158, 44, and 17 kDa). (C) Analysis on a native gel of the products of back-reactions in which native tubulin heterodimers labeled in their α -subunits were incubated with a molar excess of TBCD (to disrupt the heterodimer by sequestering the β -subunit) and TBCB (to capture released α -subunits). Note the absence of the TBCB/ α complex in the case of reactions done with the R264C mutant. Top and bottom arrows show the migration positions of the TBCB/ α -tubulin complex and native tubulin heterodimers, respectively.

Figure 4. A superabundance of TBCB enhances productive folding of the R264C α -tubulin mutant. Analysis on native gels of the products of CCT-driven in vitro folding reactions done with wild-type or R264C mutant [35 S]methionine-labeled α -tubulin target proteins in the presence of equimolar amounts of TBCC, TBCD, TBCE (with respect to CCT), and increasing amounts of TBCB. The -fold increase in concentration of TBCB with respect to the other TBC's present in the reaction is shown. Arrows (top to bottom) show the migration position of the CCT/ α -tubulin binary complex, the TBCB/ α -tubulin complex, and native tubulin heterodimers, respectively.

result of ATP-dependent interaction with CCT or via disruption of native heterodimers are unable to stably interact with TBCB.

Enhanced R264C Mutant Heterodimer Production in the Presence of an Excess of TBCB

We reasoned that the failure of the R264C mutant α -tubulin to yield the characteristic TBCB/ α -tubulin intermediate might reflect a reduced affinity of the mutant polypeptide for the TBCB chaperone, and that this reduced affinity might be compensated in tubulin heterodimer assembly reactions containing higher concentrations of TBCB. To test this idea, we repeated our CCT-driven forward folding reactions by using increasing relative concentrations of TBCB. In reactions done with wild-type target protein, the inclusion of increasing amounts of TBCB did not enhance the yield of native heterodimers; instead, with a rising stoichiometric excess of TBCB over TBCC, TBCD, and TBCE, the reaction products contained an enhanced abundance of the TBCB/ α -tubulin intermediate (Figure 4A). In contrast, parallel reactions done with the R264C target protein did result in the production of a slightly increased yield of heterodimers as a function of a greater abundance of TBCB, with no evidence of a TBCB/ α -tubulin intermediate (Figure 4B). The material migrating as heterodimer in these reactions was competent to assemble into microtubules as shown by its ability to cycle with native brain microtubules through successive cycles of

polymerization and depolymerization in vitro (data not shown). The ability of a superabundance of TBCB to contribute to an enhanced yield of native R264C mutant-containing heterodimers is consistent with the notion that a consequence of the mutation is a lowered affinity for interaction with TBCB, with a resulting compromise in the efficiency of productive heterodimer assembly.

Effect of R264C on α -Tubulin Structure

We investigated the implications of the R264C mutation in terms of the known structure of α -tubulin. The location of R264 places it at a loop between helix 8 and strand 7 of α -tubulin such that the arginine side chain forms a hydrogen bond with the carbonyl oxygen of residue D424 in the C-terminal helix (helix 12) (Figure 5A). Mutation of R264 to a cysteine residue would result in a disruption of this interaction, destabilizing the tertiary structure formed between the C-terminal α -helix and the R264 loop and possibly also the local structure of the loop itself. We reasoned that such a destabilization might lead to a decrease in overall structural stability, and that this might be reflected in an enhanced susceptibility of the mutant protein to proteolytic digestion. To test this prediction, we prepared [35 S]methionine-labeled wild-type and R264C mutant protein by translation in reticulocyte lysate, and we subjected the reaction products to limited digestion with proteinase K. The result of this experiment showed that mutant-containing heterodimers were indeed more sensitive to proteolytic attack, with the more rapid appearance of a degradation product a few kilodaltons smaller than the intact protein (Figure 5B). This could reflect cleavage of a more exposed C-terminal domain in the R264C polypeptide.

Expression of the R264C Mutation In Vivo

We examined the consequences of expressing the R264C mutant α -tubulin in vivo. TUBA1A differs from other α -tubulin isotypes by as little as a single conservative amino acid substitution; therefore, it is not possible to generate antisera that can distinguish TUBA1A from other highly homologous α -tubulin isotypes upon transfection into cultured cells. Moreover, because TUBA1A is universally expressed, we required a means to distinguish the transgene from its endogenously expressed counterpart. We therefore engineered a tag such that the transgene could be uniquely identified. Initially, we placed a FLAG tag at the C terminus of the molecule in such a way that the introduced epitope substituted for the naturally encoded C-terminal tyrosine residue. The design of this construct was based on the observation that an identically placed FLAG tag yielded het-

Arrow shows the location of intact α -tubulin. Migration of molecular mass markers is shown at the left.

Figure 5. Predicted effect of the R264C mutation on the tertiary structure of α -tubulin and its susceptibility to proteolysis. (A) Ribbon presentation of the region around R264 in α -tubulin. Helices are shown as cylinders and β strands as arrowed sheets. The side chain of R264 (shown as sticks) can form a hydrogen bond (black dots) with the carbonyl oxygen of D424 (sticks) on the C-terminal α -helix (arrowed). This hydrogen bond may stabilize the tertiary structure of this region. (B) Analysis by SDS-PAGE of tubulin heterodimers containing wild-type or R264C mutant α -tubulins generated by translation in vitro and incubated for the times shown in the figure with proteinase K.

erodimers that were polymerization competent in vitro and in vivo both in the case of the wild-type mouse homologue of TUBA1A (*tuba1*) and in the case of a mutation (S140G) in this gene (Keays *et al.*, 2007). As expected, we found that a C-terminal tag placed in this manner had no effect on the ability of wild-type TUBA1A tubulin to incorporate into microtubules upon transfection into HeLa cells. However, we were surprised to discover that the corresponding tagged R264C mutant protein completely failed to incorporate into microtubules (data not shown). This result was particularly puzzling because heterodimers containing the untagged mutant α -tubulin were clearly polymerization competent in vitro (Figure 1C). To resolve this apparent paradox, we engineered FLAG-tagged constructs in which we deleted sequences encoding 11 amino acids from the C-terminus of the α -tubulin polypeptide. The rationale behind this approach was based on the fact that up to 12 C-terminal amino acids of α -tubulin can be removed without compromising either assembly of the heterodimer or its incorporation into microtubules in vivo (Gu and Cowan, 1989), whereas removal of at least some of this highly acidic C-terminal domain might be expected to compensate for the acidic charges conferred by the FLAG epitope, which itself contributes a net charge of -3 .

To ensure that these C-terminally truncated FLAG-tagged molecules behaved in the same manner as their unmodified counterparts, we compared their properties in in vitro translation and in CCT-mediated folding reactions. Consistent with the behavior of the corresponding untagged proteins, we found that the yield of mutant product migrating as tubulin heterodimer was reduced relative to the wild-type counterpart by a factor of ~ 5 upon expression in rabbit reticulocyte lysate (Figure 6A). The wild-type and mutant C-terminally truncated cell-free translation products both cycled efficiently through successive cycles of polymerization and depolymerization with native brain tubulin (Figure 6B). Moreover, in CCT-mediated in vitro folding reactions, the wild-type (but not the R264C mutant) protein yielded the characteristic TBCB/ α -tubulin intermediate in reactions done in the presence of TBCB (Figure 6C). Thus, removal of 11 residues from the C terminus and substitution with a FLAG epitope had no significant influence on the in vitro behavior of wild-type or mutant α -tubulins compared with intact, untagged molecules. We therefore expressed the FLAG-tagged C-terminally deleted sequences by transfection in HeLa cells, and we examined their microtubules by double label immunofluorescence by using an anti-FLAG antibody (to detect the transgene) and an anti- β -tubulin antibody (to detect the overall microtubule network). We found that tagged C-terminally truncated wild-type and R264C mutant α -tubulin both incorporated into interphase and mitotic HeLa cell microtubules in vivo (Figure 6D). We conclude that the R264C mutation does not preclude assembly of the α -tubulin polypeptide into heterodimers, and that these heterodimers are competent to copolymerize into microtubules. We further infer that, in the R264C mutant, addition of a FLAG tag at the C terminus has a disruptive effect on heterodimer formation as a result of the addition of excessive negative charge, but that this effect can be compensated by removal of negatively charged residues that are not essential for heterodimer assembly or incorporation into microtubules in vitro or in vivo.

Dynamic Behavior of Microtubules Containing the R264C Mutation

To assess the effect that heterodimers containing the R264C mutation might have on microtubule dynamics, we exam-

Figure 6. Expression of the FLAG-tagged R264C mutation in vitro and in vivo. (A) Analysis by native PAGE of the products of cell-free translation reactions programmed for the expression of C-terminally truncated FLAG-tagged wild-type and R264C mutant TUBA1A sequences. Parallel reactions done using the corresponding unmodified sequences are marked with an asterisk. Top and bottom arrows show the migration positions of the CCT/ α -tubulin binary complex and native tubulin heterodimers, respectively. Note the reduced yield of heterodimers in the case of reactions done with the R264C mutant, and the slightly reduced electrophoretic mobility of tagged C-terminally truncated heterodimers due to an overall reduction in negative charge. (B) Copolymerization of the C-terminally truncated translation products shown in A with native bovine brain microtubules. Aliquots taken after successive rounds of polymerization and depolymerization and containing equal amounts of total protein were analyzed by native gel electrophoresis. (C) Analysis by native PAGE of the products of CCT-mediated folding reactions done in the presence of TBCB. Note the absence of the TBCB/ α -tubulin complex in reactions done with the R264C mutant target protein. (D) Expression of wild-type and R264C mutant TUBA1A in vivo. Constructs identical to those used in the experiments shown in A and B were used to drive the expression of wild-type and R264C mutant α -tubulin by transfection into HeLa cells; these were examined by immunofluorescence by using an anti-FLAG antibody (visualized in green) to detect the transgene and an anti- β -tubulin antibody (visualized in red) to detect the overall microtubule network. Mitotic cells expressing the transgene are shown as insets. Bar, 10 μ m.

ined microtubule behavior in cells expressing the mutant protein. To do this, we transfected cultured cells with the wild-type and mutant C-terminally deleted FLAG-tagged constructs that we established as assembly and polymerization competent in vivo. Thirty-six hours posttransfection, the cells were treated with the microtubule-depolymerizing drug nocodazole, restored to drug-free medium, fixed at various intervals, and examined by immunofluorescence by using anti-FLAG and anti- β -tubulin antibodies to determine the rate of growth of new microtubules. The result of these experiments is shown in Figure 7. We found no obvious difference in the rate of microtubule growth in asters emanating from centrosomes between tagged wild-type and R264C mutant TUBA1A sequences. However, we noted a

Figure 7. Dynamic behavior of microtubules containing the R264C mutation. HeLa cells were transfected with constructs engineered for the expression of C-terminally deleted FLAG-tagged wild-type and R264C mutant TUBA1A (see text). Thirty-six hours posttransfection, the cultures were treated with nocodazole and restored to drug-free medium. At the times indicated in the figure, cells were fixed and examined by immunofluorescence with an anti-FLAG antiserum (to visualize the transgene; green) and an anti- β -tubulin antibody (to visualize the overall microtubule network; red). Bar, 10 μ m.

higher diffuse cytosolic signal and fewer well-resolved cytoplasmic microtubules that were not anchored at the centrosome in cells expressing the R264C transgene; this was particularly evident at intermediate time points (e.g., 3.5 min) of recovery from nocodazole. These data suggest that heterodimers containing the R264C mutation may be less efficiently incorporated into microtubules than their cognate wild-type counterparts upon copolymerization with endogenously expressed tubulins *in vivo*.

DISCUSSION

Compromised Folding of R264C Mutant α -Tubulin

The discovery of mutations in one of the human genes that encode α -tubulin as a cause of lissencephaly/pachygyria expands the list of genes in which mutations can cause these diseases. Some of these genes (e.g., *TUBA1A*, *LIS1*, and *DCX*) are involved either directly or indirectly in contributing to microtubule behavior. Here, we show that one of the pachygyria-causing α -tubulin mutations, R264C, results in several defects in the *de novo* tubulin heterodimer assembly pathway. This pathway involves several chaperone proteins. Prefoldin, a multisubunit complex assembled from six subunits in the range 14–23 kDa, functions by binding to nascent α - and β -tubulin polypeptides and delivering them to the cytosolic chaperonin CCT (Hansen *et al.*, 1999; Spiess *et al.*, 2004). The chaperonin provides a locally sequestered folding environment in which tubulin polypeptides can avoid becoming kinetically trapped in off-pathway configurations, and yields quasinative folding intermediates (termed I_Q) defined by the formation of a native (N-site) GTP-binding pocket as a result of one or more cycles of iterative annealing within the chaperonin cavity (Tian *et al.*, 1995a). Notwithstanding their acquisition of bound GTP, these I_Q intermediates are incapable of assembly into polymerization-com-

petent heterodimers. For this to occur, they must first interact with a series of downstream chaperones (termed cofactors, or TBCA-E). TBCA and TBCD interact with β -tubulin folding intermediates, whereas TBCB and TBCE interact with α -tubulin folding intermediates. The TBCD/ β -tubulin and TBCE/ α -tubulin complexes associate to form a supercomplex that constitutes a tubulin heterodimer assembly machine (Tian *et al.*, 1997); interaction of this supercomplex with TBCC results in the triggering of GTP hydrolysis by β -tubulin. This latter reaction results in the release of the newly formed heterodimer (Cowan and Lewis, 2002) (Figure 1A).

We found no significant difference in overall yield between wild-type and mutant α -tubulin polypeptides upon translation *in vitro* and analysis of the products under denaturing conditions (Figure 1B). Conversely, the yield of native assembly competent mutant-containing heterodimers was lower by a factor of ~ 5 compared with a control reaction done with wild-type α -tubulin (Figure 1C). It follows that there must be one or more compromised interactions in the complex chaperone-dependent pathway leading to *de novo* tubulin heterodimer formation.

We identified two steps in the folding pathway in which the R264C mutation behaved differently from its wild-type counterpart. First, in the essential ATP-dependent interaction between unfolded polypeptides and CCT, the rate at which GTP-containing I_Q intermediates are generated is much lower in the case of the mutant protein compared with the wild-type protein (Figure 2, A and C). This is unlikely to be a structural effect of the mutation on the GTP binding pocket itself, because R264 is located far from the N-site. Consistent with this conclusion, pulse chase experiments showed wild-type and mutant I_Q intermediates to have indistinguishable half-lives (Figure 2, B and D). An effect of TBCB on the rate of generation of productive I_Q intermedi-

ates can also be ruled out, because I_Q production can be uncoupled from the action of cofactors (including TBCB) without affecting the yield of native tubulin heterodimers (Tian *et al.*, 1995a). The reduced yield of mutant I_Q molecules generated via interaction with CCT could, therefore, reflect either a lower affinity of the mutant target protein for the chaperonin, or a reduced efficiency with which mutation-containing intermediates undergoing cycles of ATP-dependent binding to and release from the chaperonin acquire a native GTP binding pocket. In either event, the R264C mutation results in a reduction of the rate at which potentially productive quasistative α -tubulin folding intermediates can form via iterative cycles of annealing of the target protein with the chaperonin.

Second, in reactions done in vitro using purified components, we found that the R264C mutant α -tubulin protein failed to yield the characteristic TBCB/ α -tubulin folding intermediate in either ATP-driven folding reactions containing CCT and TBCB (Figure 3, A and B) or in back-reactions supplemented with TBCB in which native tubulin heterodimers were disrupted by interaction with TBCD (Figure 3C). The efficient de novo production of tubulin heterodimers in vitro is absolutely dependent on the presence of TBCB (Tian *et al.*, 1997). The failure of R264C mutant α -tubulin to interact stably with TBCB, together with the reduced rate (and hence efficiency) with which CCT produces R264C quasistative GTP-containing intermediates, explains the relatively poor translational yield of polymerization competent mutant-containing heterodimers. Nonetheless, we found that we could generate some assembly competent mutant α -tubulin-containing heterodimers in vitro in the presence of TBCB-E, and that the yield of mutant-containing heterodimers generated in these reactions could be enhanced by supplying a large excess of TBCB (Figure 4). Thus, the mutant protein may still be capable of a functional interaction with TBCB, although with a much lower affinity compared with its wild-type counterpart.

Role of TBCB

Among the various chaperone proteins that participate in de novo tubulin heterodimer formation, genetic experiments with model organisms have shown that TBCB, TBCC, TBCD, and TBCE are all essential to life in higher eukaryotes (Grishchuk and McIntosh, 1999; Cowan and Lewis, 2002; Radcliffe and Toda, 2000; Steinborn *et al.*, 2002). It makes sense, therefore, that mutations resulting in compromised functioning of these proteins would result in microtubule phenotypes. In TBCE, for example, deletion mutations have been described in mouse (Martin *et al.*, 2002) and in humans (Parvari *et al.*, 2002) that lead to microtubule defects. No examples of inherited mutations in TBCB are known. However, there is persuasive evidence that TBCB plays an important role in neuronal development: induced perturbations in the level of TBCB in neurons results in either longer axons (upon TBCB depletion) or abnormalities in growth cone microtubules (upon TBCB overexpression) (Lopez-Fanarraga *et al.*, 2007) that are reminiscent of neuronal changes in giant axonal neuropathy, an autosomal recessive disorder caused by mutation in the gene encoding gigaxonin (Bomont *et al.*, 2000). The molecular mechanisms underlying this disease are not well understood, but it has been shown that gigaxonin interacts with TBCB and controls its degradation through the ubiquitin-proteasome pathway (Wang *et al.*, 2005). There is also evidence that microtubule dynamics are influenced via the p21-activated kinase 1-induced phosphorylation of TBCB (Vadlamudi *et al.*, 2005). Together with our demonstration that the pachygyria-causing R264C mutation results in a reduced affinity of the mutant polypeptide for TBCB,

these data underscore the importance of TBCB in contributing to tubulin biosynthesis and in influencing microtubule behavior.

Structural Implications

TBCB possesses two distinct domains: an N-terminal ubiquitin-like (Ubl) domain and a C-terminal domain containing a conserved glycine-rich cytoskeletal-associated protein (CAP-Gly) domain (Tian *et al.*, 1997; Lytle *et al.*, 2004). The Ubl domain of TBCB is considered to contain an α -tubulin interacting surface because in *Schizosaccharomyces pombe*, the Ubl domain of the TBCB orthologue Alp11^B is essential for viability (Radcliffe and Toda, 2000; Lytle *et al.*, 2004). Moreover, the Ubl domain of TBCE, which (like TBCB) is an α -tubulin binding component in the tubulin heterodimer assembly pathway (Figure 1A), is required for this chaperone's activity (Tian *et al.*, 2006; unpublished observations). The C-terminal domain of TBCB (which is not essential for viability in *S. pombe*) may also contribute to α -tubulin binding, because a CAP-GLY domain is present in many proteins, including several involved in modulating plus-end microtubule dynamics (reviewed in Carvalho *et al.*, 2003), and this domain contains a conserved motif (GKNDG) that is responsible for targeting to the extreme C-terminal EEY/F motif present in α -tubulin (Weisbrich *et al.*, 2007). However, the 10 carboxy-terminal residues of α -tubulin are structurally disordered (Nogales *et al.*, 1999), and they are identical in the wild-type and R264C polypeptides. Moreover, the CAP-Gly domain of TBCB lacks the highly basic groove present in proteins that bind to the C terminus of α -tubulin (Li *et al.*, 2002; Mishima *et al.*, 2007). Molecular modeling analyses (data not shown) of the predicted interaction between α -tubulin and the N-terminal Ubl domain of TBCB suggest that docking occurs at the region around the loop in α -tubulin that contains R264, consistent with the observation that the mutant protein fails to stably interact with TBCB. However, it is possible that there is an additional (and perhaps cooperative) contribution to α -tubulin binding via the C-terminal CAP-Gly domain.

Expression of R264C Tubulin In Vivo

We found that the ability of a FLAG-tagged form of the R264C mutant protein to assemble into polymerization competent heterodimers was dependent on the positioning of the tag. Thus, addition of the tag close to the authentic C terminus resulted in a protein that failed to yield productively folded heterodimers in vitro or in vivo, even though the corresponding wild-type protein tagged in an identical manner folded to the native state. Conversely, addition of the identical tag to a carboxy-terminally truncated form of either the wild-type or mutant protein yielded assembly competent heterodimers. The highly acidic 12 C-terminal amino acids of α -tubulin are not required for functional heterodimer formation (Gu and Cowan, 1989), and they are structurally disordered (Nogales *et al.*, 1999). It therefore seems likely that the R264C mutation renders the protein more structurally sensitive to overall charge changes in the flexible carboxy-terminal region.

On expression by transfection in HeLa cells, a C-terminally truncated FLAG-tagged form of the R264C mutant α -tubulin became incorporated into interphase and mitotic spindle microtubules in a manner that was indistinguishable from parallel controls done with the wild-type protein (Figure 6). This is despite the failure of the R264C mutant to stably interact with TBCB in vitro (Figure 3). We also found no significant difference in the rate of growth of centrosomally anchored microtubules in HeLa cells that had been

transfected with sequences encoding either wild-type or R264C mutant TUBA1A, although there was a relatively high diffuse signal in cells expressing the mutant sequence during early times of recovery from drug-induced depolymerization (Figure 7). These data suggest that the R264C mutation might affect the efficiency with which heterodimers can be assembled into microtubules *in vivo*, although we note that these experiments were done under transient transfection conditions using a powerful (CMV-derived) promoter such that the level of expressed protein would be much higher than that encountered in the developing brains of afflicted individuals. A reduction in polymerization efficiency could reflect structural changes in α -tubulin resulting from the mutation, for example via destabilization incurred as a result of loss of the hydrogen bond interaction between residues R264 and D424. However, we cannot discount any effects conferred by addition of the C-terminal FLAG tag.

TUBA1A and its homologues in other vertebrate species are dominantly expressed in differentiating neurological cells, especially during post-natal development (Lewis *et al.*, 1985; <http://www.ncbi.nlm.nih.gov/UniGene/>, follow "Expression Profile" link). Although other α -tubulin isoforms (e.g., TUBA2, TUBA6) are also expressed in brain tissue, the TUBA1A α -tubulin isoform is likely to be a major contributor of α -tubulin to the overall tubulin heterodimer pool in migrating cells, especially during the critical period of neuronal migration upon which proper cortical lamination depends. A paucity of assembly competent heterodimers resulting from compromised interactions among components of the heterodimer assembly pathway such as those described here could result in a limiting abundance of neuronal microtubules, and this in turn could affect neuronal migration events. We predict that other neuronal migration disease-causing mutations in α - or β -tubulins will also be characterized either by defects in the heterodimer assembly pathway such as those described here, or by a failure to interact with any of a variety of effectors that are required for the proper modulation of microtubule behavior during neuronal migration.

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