THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 44, Issue of November 3, pp. 34442–34450, 2000

Printed in U.S.A.

Patient Mutations in Doublecortin Define a Repeated Tubulin-binding Domain*

Received for publication, August 4, 2000
Published, JBC Papers in Press, August 16, 2000, DOI 10.1074/jbc.M007078200

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Doublecortin (DCX) missense mutations are found in two clusters in patients with defective cortical neuronal migration. Although DCX can function as a microtubule-associated protein (MAP), the potential relationship between its MAP activity and neuronal migration is not understood. Here we show that the two clusters of patient mutations precisely define an internal tandem repeat. Each repeat alone binds tubulin, whereas neither repeat is sufficient for co-assembly with microtubules. The two tandem repeats are sufficient to mediate microtubule polymerization, and representative patient missense mutations lead to impaired polymerization both *in vitro* and *in vivo* as well as impaired microtubule stabilization. Furthermore, each repeat is predicted to have the secondary structure of a β-grasp superfold motif, a motif not found in other MAPs. The patient mutations are predicted to disrupt the structure of the motif, suggesting that the motif may be critical for the DCX-tubulin interaction. These data provide both genetic and biochemical evidence that the interaction of DCX with microtubules is dependent upon this novel repeated tubulin-binding motif.

Insight into specific functions and requirements for microtubules in diverse biological processes have recently been aided through positionally cloned genes such as *doublecortin* (*DCX*)[1] that share no sequence similarity to known microtubule-associated proteins. Mutations in *DCX* lead to the human disorder double cortex and X-linked lissencephaly (1, 2), which appears to be due to a primary defect in cortical neuronal migration (3), resulting in epilepsy and mental retardation (4). In lissencephaly (*lissos* means smooth) the normally gyrated six-layered cortex is replaced by a smooth four-layered cortex, whereas in double cortex there is a normal-appearing outer cortex and a second layer of cortical neurons in the subcortical white matter.

* This work was supported by National Institutes of Health Grants RO1 NS 38097 and PO1 NS39404 (to C. A. W.), by NINDS Neurological Sciences Academic Development Award 5K12NS01701-05, by the Department of Neurosciences at the University of California, San Diego, by a Junior Investigator Grant from the Epilepsy Foundation, and by funds from the Searle Scholars Program (to J. G. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: DCX, doublecortin; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; AA, amino acids; MTOC, microtubule-organizing center.

The *DCX* gene was recently shown to encode for a microtubule-associated protein, based on its co-localization and co-assembly with microtubules and its pronounced effect on microtubule polymerization *in vitro*. Furthermore, overexpression of *DCX* in neuronal cells leads to pronounced microtubule polymerization and stabilization *in vivo* (5–7). However, because of its novel sequence, the mechanism of the interaction of DCX with microtubules is completely unknown.

Patient missense mutations in *DCX* suggest that there may be two critical domains, raising the possibility that these domains may be important for the interaction of DCX with microtubules. Over 30 *de novo* mutations have been identified (8–10), largely representing either nonsense mutations or missense mutations. Interestingly, although the nonsense mutations occur randomly throughout the protein, all of the identified missense mutations have been identified in two tightly clustered regions (8), suggesting that these two regions are critical functional domains. These two critical domains bear no resemblance to any of the known microtubule-interacting domains of other microtubule-associated proteins, suggesting that if these critical domains are involved in the interaction of DCX with microtubules, they may define a new microtubule-association domain and provide insight into the function of DCX in neuronal migration. Here we show that the patient missense mutations define an internal repeat within DCX and that this repeated domain represents a new tubulin-binding motif. Because the patient mutations in DCX lead to defective interactions with microtubules, this suggests that DCX-tubulin interactions are critical for the role of DCX in neuronal migration.

MATERIALS AND METHODS

DCX Fragment Construction—Constructs containing fragments of DCX were assembled by polymerase chain reaction amplification with proofreading *Taq* polymerase from a full-length *DCX* clone (6) using the following pairs of primers: 1F, TCGAGGTCGACCATGGAACTTCATTTTGGACAC; 47F, AGTAATGAGAAGAAAGCCAAG; 140R, CTTGGTGTACTCCACCTTTTTAAAG; 150F, TCGAGGTCGACCACATCTGCCAATATGAAAG; 170F, GCCAGGGAGAACAAGGACTTTG; 171R, ACTAGTACCTGGCCTGTGCACTGTTGCTGC; 260R, AGCATAGCGAAATTTTTCAG; 268F, GAAAATGAATGCCGAGTCATG; 268R, ACTAGTATTCATCCAGAGAAAAATCATCC; and 361R, CATGGAATCACCAAGCGAGTC. Each polymerase chain reaction amplified product was cloned into the Zero Blunt TOPO polymerase chain reaction cloning vector (Invitrogen, Carlsbad, CA), according to the manufacturer's suggestions. Inserts were then removed by restriction digest with *Eco*RI and gel purified. Each insert was then shuttled into both the pET-28a (+) vector (Novagen, Madison, WI), and the pcDNA3.1/HisA vector (Invitrogen) previously digested with *Eco*RI was treated with calf alkaline phosphatase. The open reading frame of each construct was sequenced in its entirety to confirm proper clone construction.

Introduction of Patient Mutations into Wild Type DCX—Full-length

DCX in both pET-28a (+) and pcDNA3.1/HisA was mutagenized using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) and the primers R59HF, GGTACGTTTCTACCACAATGGGGACCGC; R59HR, GCGGTCCCCATTGTGGTAGAAACGTACC; R89GF, GCTGGCTGACCTGACGGGATCTCTGTCTGAC; R89GR, GTCAGACAGAGATCCCGTCAGGTCAGCCAGC; R192WF, GGGGTGAAGCCTTGGAAGGCTGTGCGTGT; R192WR, TGTGCGTGTCGGAAGGTTCCGAAGTGGGG; T203RF, GCTTCTGAACAAGAAGAGAGCCCACTCTTTTC; and T203RR, GTTTTCTCACCCGAGAGAAGAACAAGTCTTC. The open reading frame of each construct was sequenced in its entirety to confirm proper clone construction.

Expression of Wild Type, DCX Fragments, and Patient DCX Mutations in COS-7 Cells—COS-7 cells were transiently transfected with each of the pcDNA3.1 constructs using Superfectamine (Qiagen, Chatsworth, CA), according to the manufacturer's recommendations. Transfected cells were maintained for 2–3 days either on microscope slides for immunofluorescence or on 150-mm dishes for protein production. To assay the ability of wild type *versus* mutant protein to polymerize or stabilize microtubules *in vivo*, transfected cells were exposed to 4 °C for 15–30 min to allow for depolymerization of microtubules, followed by exposure to 37 °C for 2, 4, or 10 min to allow for repolymerization of microtubules. For immunofluorescence, cells were rinsed with phosphate-buffered saline and fixed with 0.5% glutaraldehyde/0.1% Triton X-100 in 80 mM potassium PIPES (pH 6.8), quenched in 1 mg/ml $NaBH_4$ in phosphate-buffered saline, blocked with 1% bovine serum albumin and 5 mM lysine in phosphate-buffered saline for 1 h, labeled with anti-Express mouse monoclonal antibody (Invitrogen) at 1:120 (to detect the DCX fusion protein) and anti-tyrosinated α-tubulin rat monoclonal antibody at 1:200 (clone YL 1/2, Harlan Bioproducts, Indianapolis, IN), followed by fluorescein isothiocyanate-labeled anti-mouse (1:200) and rhodamine-labeled anti-rat (1:100) antibodies (Jackson ImmunoResearch, West Grove, PA), and examined using confocal microscopy. Approximately five cells were photographed for each experimental condition and analyzed visually. For protein isolation, cells were lysed in 40 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 1 mM EGTA, 0.7 M sucrose, 150 mM NaCl, and protease inhibitors, incubated for 30 min at 4 °C, and cleared by centrifugation to produce whole cell lysates.

DCX Fragment Microtubule Co-assembly Assay—To evaluate for passive co-assembly of each DCX fragment into microtubules, purified tubulin was taxol-assembled in the presence of the whole cell lysates from transfected COS-7 cells. Lysates were incubated with 700 μg of phosphocellulose-purified tubulin, and 10 μM taxol in 700 μl of PEM-GTP buffer (100 mM sodium PIPES (pH 6.6), 1 mM EGTA, 1 mM $MgSO_4$, 1 mM GTP) at 37 °C for 30 min to allow for microtubule assembly, and centrifuged at 25,000 rpm at 35 °C for 30 min to isolate the microtubule pellet. The pellet was washed with warm PEM-GTP buffer, boiled in sample buffer, and analyzed by SDS-PAGE Western for the presence of the DCX fragment by probing with the anti-Express antibody at 1:400 and detection by chemiluminescence. The Western blot was digitized, and band intensity was quantitated by densitometry (ImageQuant software; Molecular Dynamics, Sunnyvale, CA). Results were standardized to the amount of fusion protein present in each of the whole cell lysates. Results reported are averaged from an $n = 2$.

Production of Purified Protein—Each of the pET-28a (+) constructs was used to produce recombinant His_6-tagged protein in BL21 DE3 *Escherichia coli* (Novagen), according to the manufacturer's recommendations. Proteins were affinity-purified using HisBind resin (Novagen), followed by dialysis in 100 mM HEPES (pH 7.5), 200 mM NaCl, 10 mM $MgCl_2$ overnight at 4 °C, and concentrated to approximately 2 mg/ml (Untrafree Biomax concentrators; Millipore, Bedford, MA).

In Vitro Binding Assay—The cells from 50-ml cultures containing each of the His_6-DCX fragments were isolated, sonicated, and cleared by centrifugation as described. Five μg of phosphocellulose-purified tubulin and 1 mM GTP was added to each sample, and each fragment was isolated by affinity column purification (as above) and subsequently analyzed for both the presence of the purified DCX fragment by Coomassie stain and the presence of co-purifying tubulin by SDS-PAGE Western analysis using both anti α- and β-tubulin monoclonal antibodies (Sigma).

Polymerization Assay—Each purified DCX fragment and patient mutation was assessed for its ability to polymerize phosphocellulose-purified tubulin using the light scattering assay (11) as described previously (6). Each reaction contained 100 μg of phosphocellulose-purified tubulin and approximately 1.75 μM DCX or DCX fragment in 100 μl PEM-GTP buffer. Thus, the ratio of tubulin to DCX was kept at approximately 15:1 molar ratio, which was previously determined to lead to optimal DCX-induced microtubule polymerization (6). Additionally, at the completion of the turbidity assay, a microtubule pellet was isolated by centrifugation and its mass was determined, to provide an additional assessment of the microtubule polymerization. Results reported are averaged from an $n = 2$ for each experiment.

RESULTS

DCX Contains an Internal Repeat Defined by Disease-related Missense Mutations—Based upon the published microtubule functions of DCX and the identified clusters of patient mutations, we considered first whether DCX may contain a previously described microtubule-association domain (12–17). Therefore, we searched the predicted amino acid sequence of *DCX* in the region of the patient mutations, with the goal of identifying a known microtubule-binding domain. Instead, we identified an internal repeat that is precisely outlined by these mutations, corresponding to amino acids 47–140 and 170–260 (Fig. 1). Each repeat is approximately 90 amino acids in length, with approximately 27% amino acid identity and 47% amino acid conservation between the two repeats (hereafter referred to as R1 and R2). Strikingly, amino acid substitution mutations were identified in matching amino acids between R1 and R2 (*i.e.* Arg^{59}/Arg^{186} and Gly^{100}/Gly^{223}), supporting the functional conservation between the two DCX repeats.

Two DCX Repeats Is the Minimal Domain Sufficient for Co-assembly with Microtubules—We hypothesized that like other microtubule-associated proteins, each DCX repeat may bind to microtubules and that the intact repeats may be necessary for polymerization (18–20). We also hypothesized that if this were the case, then the naturally occurring patient missense mutations should show an impaired ability to polymerize microtubules. To test whether each repeat alone is sufficient to bind to microtubules, we prepared several constructs containing either R1, R2, R1+R2, or the serine/proline-rich tail alone (Fig. 2) for either bacterial or mammalian overexpression. Additionally, several of the patient missense mutations were introduced into the wild type DCX protein.

We first evaluated for the minimal DCX fragment that is sufficient for co-assembly with microtubules. Phosphocellulose-purified tubulin was polymerized with taxol in the presence of whole cell lysates from COS-7 cells that expressed epitope-tagged wild type DCX or each of the DCX fragments, and the microtubule pellet was then analyzed for the presence of DCX or the DCX fragment by SDS-PAGE Western analysis using an antibody to the epitope tag. Although wild type DCX and each of the constructs containing both R1+R2 (AA 47–260 and 1–268) co-assembled with microtubules, none of the constructs containing a single repeat (AA 47–140, 47–171, and 170–260) co-assembled with microtubules, suggesting that two repeats is the minimal domain sufficient for microtubule co-assembly (Fig. 3*A*). Additionally, AA 268–361, containing the serine/proline-rich tail, did not co-assemble with microtubules, suggesting that the tail does not play a role in microtubule co-assembly. Interestingly, one of the constructs, AA 1–171, containing the amino-terminal 46 amino acids and the inter-repeat region in addition to R1, did co-assemble with microtubules, albeit with lower efficiency, suggesting that there may be additional motifs in these regions that may promote co-assembly with microtubules. On the other hand, one of the constructs, AA 1–268, co-assembled with microtubules 20 times more efficiently than wild type DCX, possibly indicating that the carboxyl-tail of DCX functions to negatively regulate the interaction of DCX with microtubules.

To be certain that post-translational modification of each DCX fragment is not required for binding to taxol-stabilized microtubules, this experiment was repeated using identical fragments obtained from bacterial overexpression. Again, wild type DCX and each of the constructs containing both R1+R2 (AA 47–260 and 1–268) co-assembled with microtubules,

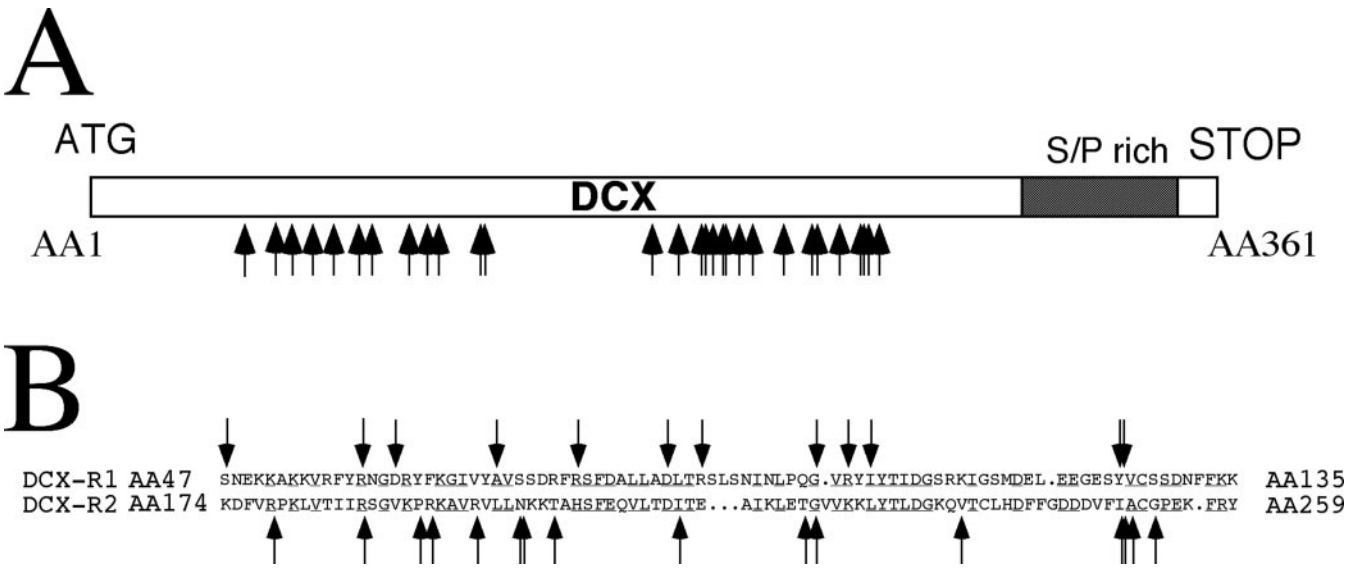


FIG. 1. **Patient mutations in DCX define an internal repeat.** *A*, patient missense mutations cluster in two regions of the open reading frame. The only recognizable domain within DCX is the serine/proline-rich tail (*S/P rich*). An *arrow* indicates the location of each of the patient missense mutations. *B*, the patient mutation clusters precisely define an approximately 90-amino acid repeat within DCX. The predicted DCX sequence from amino acids 47–135 is indicated in the *top line*, and amino acids 174–259 are indicated in the *bottom line* to demonstrate the internal repeat. The locations of the patient mutations are indicated by *arrows*, with the *top arrows* indicating mutations in the first repeat, and the *bottom arrows* indicating mutations in the second repeat. Amino acids that are identical or highly homologous between the two repeats are indicated by *underlining*. The two repeats share approximately 27% amino acid identity and 47% amino acid conservation.

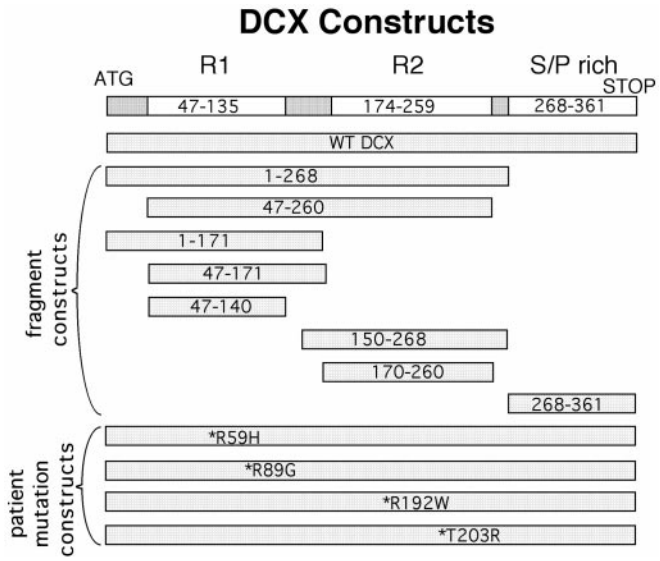


FIG. 2. **Patient constructs used to study the interaction between DCX and microtubules.** The sequence of DCX is indicated schematically by the *top bar*, with the location of the two repeats (*R1* and *R2*) and the serine/proline-rich tail (*S/P tail*). One-repeat constructs contain exclusively a single repeat (AA 47–140 and 170–260) or a single repeat plus the surrounding amino acids (AA 1–171, 47–171, and 150–268). Two-repeat constructs contain exclusively the two repeats plus the inter-repeat region (AA 47–260) or the two repeats plus the surrounding amino acids (AA 1–268). Additionally, a construct containing the serine/proline-rich tail was made (AA 268–361). Several of the patient mutations were introduced into the wild type DCX sequence, including R59H, R89G, R192W, and T203R, to study the function of these naturally occurring disease-causing alleles.

whereas fragments with a single repeat (AA 47–140 and 170–260) failed to co-assemble with microtubules, suggesting that post-translational modification of DCX is not required for co-assembly with microtubules (data not shown). Fragments with a single repeat plus the inter-repeat region (AA 1–171 and 47–171) did co-assemble with microtubules, albeit with lower efficiency, again suggesting that additional motifs in these regions may promote co-assembly with microtubules.

The minimal DCX domain required for co-assembly with microtubules was also independently assessed by examining for co-localization of wild type DCX or each of the DCX fragments with cellular microtubules *in vivo*. Each of the DCX constructs was transiently transfected into COS-7 cells, and the localization of the epitope-tagged fusion protein was determined in relationship to microtubules by two color fluorescent confocal microscopy. Each of the constructs containing at least R1+R2 co-localized with cellular microtubules (Fig. 3*B*), supporting the co-assembly data. Again, a single repeat was not sufficient for co-localization with cellular microtubules, suggesting that a single repeat does not bind to microtubules nor co-assemble with microtubules.

A Single DCX Repeat Can Bind to Tubulin—We considered the possibility that a single DCX repeat may bind to tubulin but not to assembled microtubules. Therefore, we utilized a modified *in vitro* binding assay to test whether purified tubulin dimers can co-purify with each of the DCX repeats. His-tagged wild type DCX or each of the His-tagged DCX fragments was expressed in *E. coli*, and after bacterial lysis and clearing by centrifugation, GTP-bound phosphocellulose-purified tubulin was added to each of the samples. The His_6 containing proteins were purified from the lysates using affinity purification, and each sample was analyzed for the presence of co-purifying tubulin by SDS-PAGE Western analysis. Wild type DCX and each of the DCX fragments containing at least a single DCX repeat co-purified with β-tubulin, whereas the serine/proline-rich tail did not purify with β-tubulin (Fig. 4), suggesting that a single repeat is sufficient for binding to tubulin. This blot also reacted identically with an antibody to α-tubulin (data not shown), suggesting that a single repeat binds to the α-β-tubulin dimer or a short microtubule fiber. We questioned whether constructs with two DCX repeats bound twice as much tubulin as one DCX repeat, so the results of this experiment were analyzed by band densitometry followed by standardization for the amount of purified DCX protein present in each experiment. Consistent with this hypothesis, two-repeat DCX fragments bound nearly exactly twice as much tubulin as one-repeat DCX fragments (data not shown). Taken together with the above data, this suggests that a single repeat can bind to

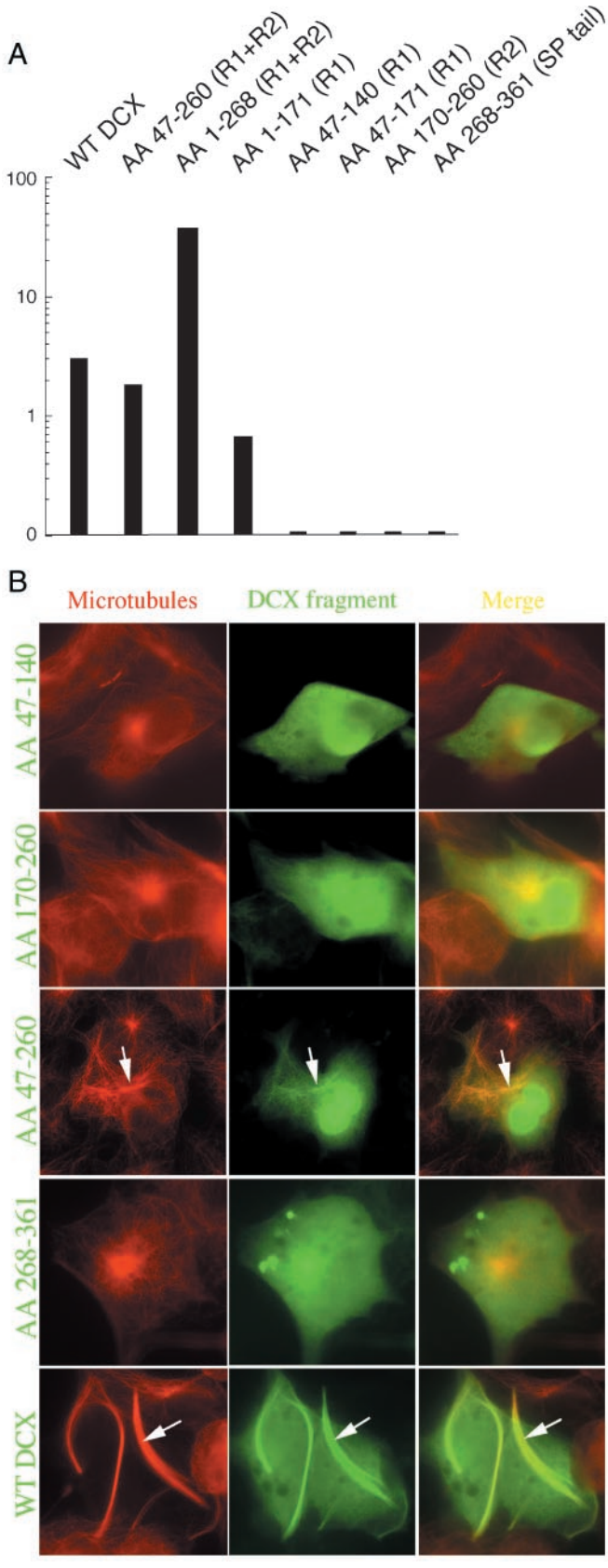


FIG. 3. **A single repeat is not sufficient for microtubule co-assembly.** *A*, co-assembly of DCX fragments with taxol-stabilized microtubules. Equivalent amounts of DCX proteins or fragments produced from transfected COS-7 cells were incubated with tubulin and induced to polymerize with taxol, the microtubule pellets were analyzed by SDS-PAGE Western for the presence of the DCX fragments and quantitated by densitometry. Each of the fragments containing two repeats (WT DCX and AA 47–260 and 1–268) co-assembled with microtubules, whereas each of the fragments containing a single repeat (AA 47–140, 47–170, and 170–260) or the S/P tail (AA 268–361) failed to co-assemble, with the exception of AA 1–171, which partially co-assembled. Results are plotted on a pseudo-logarithmic scale to simplify data interpretation. *B*, co-localization of representative DCX fragments with microtubules in cultured cells. Each of the fragment-containing constructs was transfected into COS-7 cells and analyzed by confocal microscopy for the microtubule cytoskeleton (*red*) and the epitope-tagged fragment (*green*). Neither AA 47–140 (R1), AA 170–260 (R2), nor AA 268–361 (S/P tail) co-localized with microtubules. The DCX fragment AA 47–260, (R1+R2) has overlapping expression with microtubules (*white arrow* in 47–260 indicate microtubules that are immunoreactive for this fragment), consistent with the co-assembly data. Co-localization of wild type (*WT DCX*) with microtubules is included for comparison. WT DCX co-localizes with microtubules and leads to striking microtubule bundling (*arrows* highlight bundled microtubules).

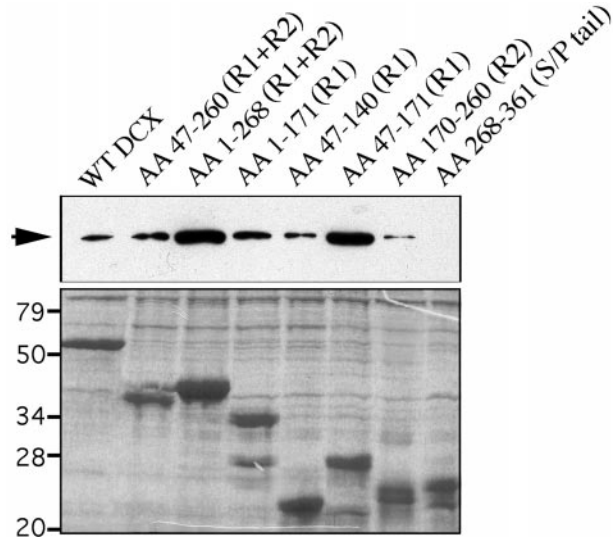
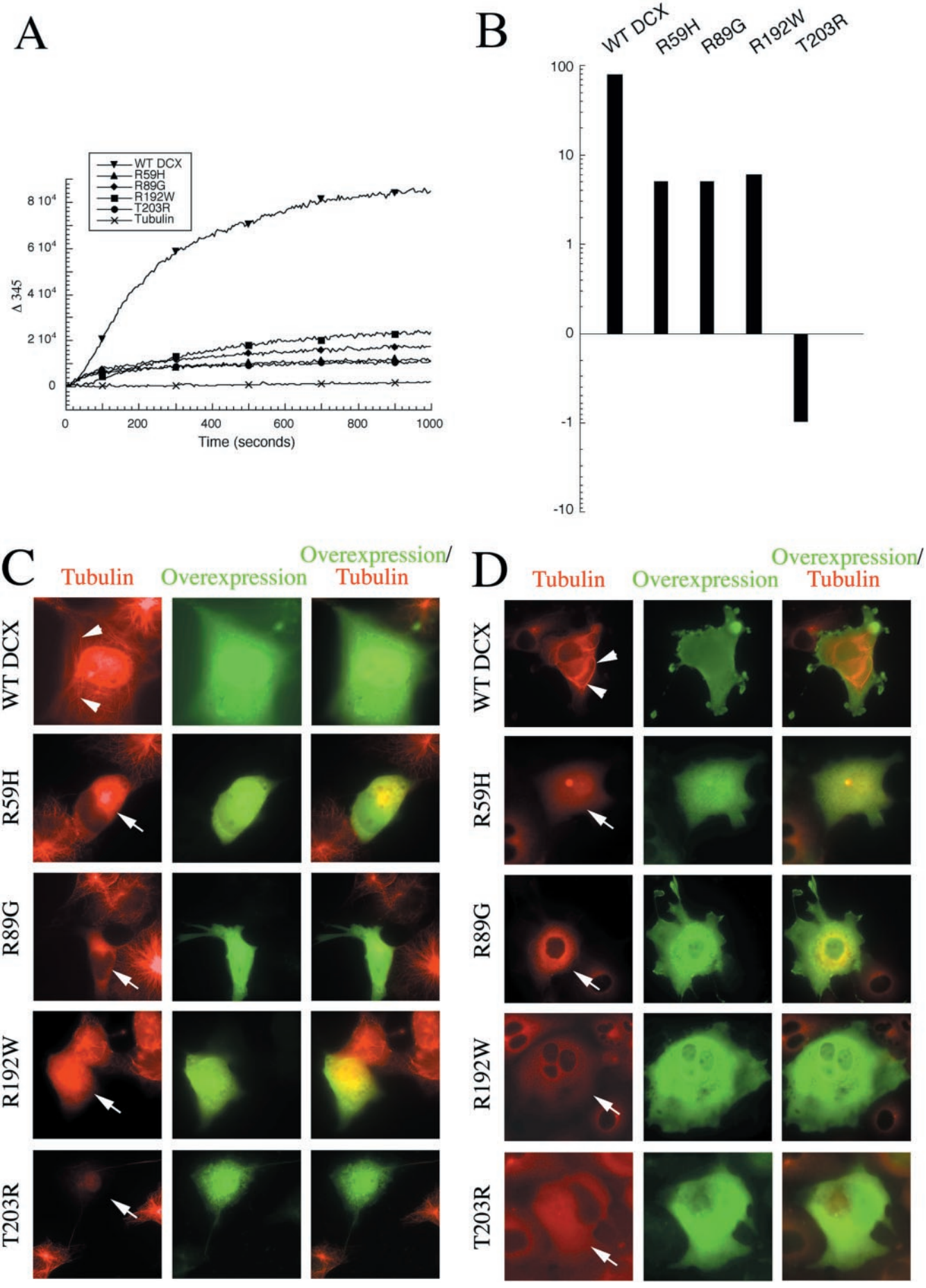


FIG. 4. **Individual DCX repeats bind tubulin.** Each of the DCX fragments was produced as a His_6 fusion *p*rotein in *E. coli*. Purified tubulin and GTP were added to each of the whole cell lysates. The His_6 fusion proteins were subsequently affinity purified using Nickel resin, and the purified protein was analyzed for the presence of co-purifying tubulin by SDS-PAGE Western using anti β-tubulin antibodies (*arrow* in *top panel* indicates β-tubulin). The blot reacted identically with α-tubulin antibodies, suggesting that each DCX repeat binds to an α−β tubulin dimer. The minimal tubulin-binding domain is one repeat, as each of the fragments contains at least one repeat bound tubulin, whereas the serine/proline-rich tail did not bind tubulin. The Coomassie-stained gel below demonstrates each of the purified DCX fragments for reference. Quantification of band intensity by densitometry suggests that two-repeat constructs bound nearly exactly twice the amount of tubulin as one-repeat constructs (data not shown). Molecular mass markers are in kDa.

tubulin but not to microtubules, and a single DCX repeat is not incorporated into a growing microtubule fiber.

Two Intact DCX Repeats Are Necessary for Microtubule Polymerization and Stabilization—Based on the finding that the naturally occurring patient mutations cluster in the two repeats and that these repeats bind tubulin, we questioned whether the patient mutations would interfere with the ability of DCX to polymerize tubulin into microtubules. Four patient mutations were chosen as representative of the patient missense mutations, two from each repeat (R59H, R89G, R192W, and T203R; see Fig. 1) because they were among the first mutations identified and occurred at well spaced intervals along each repeat. First, we tested whether DCX with these engineered patient mutations produced stable proteins in mammalian cells. Each of the constructs was transiently transfected into COS-7 cells and visualized by immunofluorescence together with co-staining for cellular microtubules. Each of the introduced patient mutations led to a stable protein that co-


A
WT DCX
R59H
R89G
R192W
T203R
Tubulin
Δ 345
8 10⁴
6 10⁴
4 10⁴
2 10⁴
0
0
200
400
600
800
1000
Time (seconds)
B
WT DCX
R59H
R89G
R192W
T203R
100
10
1
0
-1
-10
C
Tubulin
Overexpression
Overexpression/ Tubulin
WT DCX
R59H
R89G
R192W
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Tubulin
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Overexpression/ Tubulin
WT DCX
R59H
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R192W
T203R

localized with cellular microtubules (data not shown), suggesting that the deleterious nature of these patient mutations could be analyzed by testing for altered interactions with microtubules.

Each of the representative patient mutations led to significantly decreased microtubule polymerization compared with wild type DCX protein in three different polymerization assays, suggesting that two intact repeats are necessary for efficient microtubule polymerization. First, each purified DCX mutant protein was tested for its ability to polymerize tubulin using the turbidity assay. In this assay, the turbidity of a dilute tubulin solution increases as the tubulin polymerizes, as measured by a real time fluorimeter. The concentration of the tubulin in the reaction is low enough that little polymerization occurs in the absence of microtubule-polymerizing agents. In this experiment, 1.75 μM wild type DCX led to striking microtubule polymerization over a 15-min time period, as had been reported (5, 6). However, 1.75 μM DCX with any of the introduced patient mutations displayed between 10 and 25% of the polymerizing activity of wild type DCX (Fig. 5*A*), suggesting that two intact repeats are necessary for DCX-induced microtubule polymerization. Because there was some microtubule polymerization seen with mutant DCX, these results did not distinguish between whether the mutations likely function as hypomorphic alleles or complete null alleles. Therefore, higher concentrations of mutant DCX protein were tested for their ability to polymerize tubulin in the turbidity assay. 5-fold higher concentration of the R192W mutant DCX (8.75 μM) lead to significantly more polymerization (approximately 2-fold more polymerization than that observed at 1.75 μM) but still not comparable with the level of polymerization observed with wild type DCX at the lower (1.75 μM) concentration. These results suggest that the R192W mutation may be a hypomorphic allele. On the other hand, 5-fold higher concentration of the T203R mutant DCX lead to decreased overall polymerization compared with the lower concentration (data not shown), suggesting that this mutation is more likely a complete null allele. These results suggest that the interaction of mutant DCX with tubulin is defective, with some mutations retaining residual polymerizing activity, whereas others may have more severe defects in their interactions with tubulin.

As an additional measure of the ability of mutant DCX to polymerize microtubules, at the completion of the previous experiment, the mass of the microtubule pellet was measured. Consistent with the turbidity assay, DCX with any of the introduced patient mutations resulted in minimal microtubule polymerization (Fig. 5*B*) based upon the weight of the pellet compared with controls. To be certain that the results obtained with both the turbidity assay and the pelleting assay represented true polymerization and not destabilization or precipitation of tubulin, the experiment was repeated in the presence of rhodamine-labeled tubulin to directly visualize polymerized microtubules by fluorescent microscopy. Both wild type DCX and mutant DCX led to visible microtubule polymerization against a background of unpolymerized rhodamine-labeled tubulin without visible clumps of protein (data not shown), indicating that the previous assays very likely measured microtubule polymerization. As expected, there were fewer microtubules visible after polymerization with mutant DCX in this experiment, although these results were not quantitated.

The ability of mutant DCX to polymerize tubulin was also demonstrated to be defective *in vivo* in transfected cells. We hypothesized that an inability of mutant DCX to polymerize microtubules should be most evident during the recovery phase after cold temperature-induced microtubule depolymerization. Therefore, wild type DCX and each of the representative patient mutations were transfected in COS-7 cells, and after a 48-h incubation, microtubules were depolymerized by exposure to 4 °C for 30 min. After this 30-min time exposure, microtubules were verified to be nearly completely depolymerized. Cells were then rewarmed to 37 °C for 2, 4, or 10 min to allow for the initiation of microtubule polymerization. Cells were immediately fixed and processed to identify transfected cells and microtubules using two-color confocal microscopy. Untransfected cells appeared to initiate polymerization exclusively from the microtubule-organizing center (MTOC) (21), based upon the presence of asters of polymerized microtubules emanating from the perinuclear region visible at all three time intervals. Cells transfected with wild type DCX displayed polymerized microtubules throughout the cell soma and not clearly emanating from the MTOC, consistent with an effect of wild type DCX on microtubule polymerization. This was most evident at the 2-min time interval (Fig. 5*C*), whereas at later time intervals the microtubules began to take on a bundled appearance (data not shown). Cells transfected with each of the representative mutant DCX constructs demonstrated an absence of microtubule polymerization at the 2-min time interval (Fig. 5*C*), suggesting that mutant DCX impairs the ability of the cell to achieve any microtubule recovery. At later time intervals there was some polymerization evident in all transfected cells, and the differences between the cells transfected with wild type and mutant DCX decreased over the time course of the experiment.

DCX was also previously demonstrated to stabilize microtubules against cold-induced depolymerization, so we tested whether the patient mutations were associated with a defect in microtubule stabilization. Wild type DCX and each of the representative patient mutations were transfected in COS-7 cells, and after a 48-h incubation, microtubules were depolymerized by exposure to 4 °C for 15 min. Cells were immediately fixed

FIG. 5. **Patient mutations in DCX are associated with defective microtubule polymerization and stabilization.** *A*, each of the DCX patient mutations leads to significantly attenuated polymerization activity in a turbidity assay. Equimolar concentrations of each recombinant protein was incubated with purified tubulin at approximately a 1:15 ratio of DCX to tubulin, and polymerization was measured by turbidity over 15 min. Each of the mutant proteins led to minimal microtubule polymerization compared with wild type DCX. Tubulin alone served as a negative control for polymerization. *B*, each of the DCX patient mutations leads to significantly attenuated polymerization activity in a pelleting assay. At the completion of the turbidity assay, the mass of the resultant microtubule pellet was measured. Each sample was standardized with the weight of the microtubule pellet in the vehicle control set to zero. (The negative weight of the T203R sample refers to the fact that the pellet weighed less than the vehicle control pellet.) Results are plotted on a pseudo-logarithmic scale to simplify data interpretation. *C*, mutant DCX has a defect in microtubule polymerization when compared with wild type DCX *in vivo*. Each of the patient mutations was transfected into COS-7 cells and after 2 days, microtubules were depolymerized by exposure to 4 °C for 30 min, followed by microtubule repolymerization by rewarming to 37 °C for 2 min then fixed and stained. In this experiment, there was some microtubule polymerization visible in untransfected cells emanating from the MTOC, whereas overexpression of wild type DCX led to polymerization throughout the cell soma not necessarily associated with the MTOC. Microtubules are not evident in cells transfected with mutant DCX (*arrow* indicates the transfected cell in each experiment). *D*, mutant DCX has a defect in microtubule stabilization when compared with wild type DCX *in vivo*. Each of the patient mutations was transfected into COS-7 cells, and after 2 days, microtubules were depolymerized by exposure to 4 °C for 15 min then fixed and stained. In this experiment, there was complete microtubule depolymerization in untransfected cells (in the background), whereas overexpression of wild type DCX led to microtubules that were resistant to depolymerization (*arrowhead* in *top row*). Microtubules are not evident in cells transfected with mutant DCX (*arrow* indicates the transfected cell in each experiment).

and processed to identify transfected cells and microtubules using two-color confocal microscopy. Untransfected cells displayed complete or near complete depolymerization of microtubules, whereas cells transfected with wild type DCX displayed clearly visible microtubules (Fig. 5*D*). Cells transfected with each of the representative mutant DCX constructs demonstrated an absence of microtubules similar to surrounding untransfected cells, suggesting that mutant DCX has a defect in microtubule stabilization, as well as a defect in polymerization.

Two Intact Repeats Are Sufficient for Microtubule Polymerization—Because two intact repeats appear to be necessary for microtubule polymerization, we questioned whether the two repeats alone are sufficient for this polymerization. Therefore, we utilized purified wild type DCX and each of the recombinantly produced DCX fragments and tested each for its ability to polymerize microtubules in a turbidity assay. None of the single DCX repeats led to significant microtubule polymerization. However, each of the two constructs containing both intact repeats (R1+R2) (AA 1–268 and 47–260) led to significant microtubule polymerization in this assay (Fig. 6*A*), suggesting that two DCX repeats are sufficient for microtubule polymerization. The ability of each of these proteins to polymerize microtubules was significantly attenuated when compared with wild type protein, suggesting that other regions of DCX may exert polymerizing effects as well. As an additional measure of the ability of the R1+R2 constructs to polymerize microtubules, at the completion of the experiment, the mass of the microtubule pellet was measured. Again, each of the constructs containing both intact repeats (R1+R2) (AA 1–268 and 47–260) led to significant microtubule polymerization compared with constructs containing a single repeat or tubulin alone (Fig. 6*B*). Because the AA 1–268 and 47–260 constructs both contain the inter-repeat region (AA 141–169), these experiments do not evaluate for the possibility that this inter-repeat region may itself be necessary for polymerization, as has been suggested for tau (12). However, unlike tau (12), a single repeat plus the inter-repeat region cannot polymerize microtubules (constructs AA 1–171 and 47–171 do not polymerize), suggesting that the inter-repeat region does not impart significant polymerizing activity.

To be certain that the results obtained with both the turbidity assay and the pelleting assay represented true polymerization and not destabilization or precipitation of tubulin, the experiment was repeated in the presence of rhodamine-labeled tubulin as above. Polymerized microtubules were visible against a background of unpolymerized rhodamine-labeled tubulin for wild type DCX and for each of the two-repeat fragments without visible clumps of protein (data not shown), indicating that the previous assays likely measured microtubule polymerization. Rare scattered microtubules with occasional clumps of rhodamine were visible for each of the one-repeat constructs, suggesting that some precipitation of tubulin occurred in the presence of these fragments.

Each DCX Repeat May Form a β-Grasp Superfold Motif—Predictions of the secondary structure of each of the DCX repeats suggest that each may take the form of a β-grasp superfold, a structural motif shared by several proteins with unrelated sequences, and that the mutations may interfere with this structure. The PredictProtein program and the Discrimination of Protein Secondary Structure Class program predict that each repeat may form five β-sheets surrounding an α-helix, with the residues (from the first repeat) KAKKVR and KGIVYA forming two β-sheets, FRSFDALLADLTR forming an α-helix, and VRYIYTIDGS, RKIG, and YVCSSD forming three more β-sheets. Therefore, each repeat is predicted to have a $\beta_2\alpha\beta_3$ architecture. The configuration and spacing of the predicted $\beta_2\alpha\beta_3$ motif in each DCX repeat is most similar to a motif known as a β-grasp superfold (a β-sheet curled around an α-helix). β-Grasp superfold motifs have been predicted and subsequently crystallized from several proteins, including the Ras-interacting domain of c-Raf1 (22, 23) and RalGEF (24, 25) among other proteins (from Structural Classification of Proteins web site). Modeling of the predicted β-grasp superfold of DCX together with patient mutations suggests that these mutations, which largely fall at the edges of the key β-sheets or α-helices, should lead to a significant change in the structure of the β-grasp superfold, suggesting at least one possible mechanism for the deleterious nature of the patient mutations in DCX.

DISCUSSION

Here we demonstrate that the clustering of that the naturally occurring patient mutations in DCX precisely outline an internal repeat and that this repeated domain is essential for the function of DCX on microtubule polymerization. These results support the idea that the function of DCX in neuronal migration may be through polymerization of microtubules as previously proposed (5, 6), because these naturally occurring patient mutations severely disrupt the ability of DCX to polymerize microtubules. Surprisingly, a single DCX repeat can bind to tubulin but not microtubules, and a single repeat is not incorporated into a growing microtubule fiber. Finally, we demonstrate that two intact DCX repeats are necessary and sufficient for microtubule polymerization. This is the first demonstration that each of the two DCX repeats is capable of binding to tubulin and that the two repeats in tandem are sufficient for microtubule polymerization. Additionally, this is the first demonstration that mutant DCX displays a quantitative defect in microtubule polymerization. These data support and extend recently published data (26) identifying the tandem repeat within DCX and demonstrating that one of the patient mutations (Y125H) displays severing of microtubules *in vitro*.

An Internal Repeat Required for Microtubule Polymerization Defined by the Patient Mutations—Understanding the deleterious nature of the patient mutations was key to identifying a role for DCX in neuronal migration. Because the sequence of DCX is entirely novel, it was impossible to make predictions about its potential functions in neuronal migration. The clustering of patient mutations in two regions suggested that these regions are critical for the normal function of the protein, but because there is no clear biochemical role for DCX, the function of the critical domains was unclear. The possibility that DCX may function as a microtubule-associated protein was intriguing, because it suggested a potential function; however, there was no prior demonstration that the patient mutations interfered with the microtubule-based function of DCX. Our results provide strong genetic evidence that the critical function of DCX in neuronal migration is likely dependent upon an effect on polymerization of microtubules, because all of the missense mutations occur within the two repeats, and these mutations interfere with DCX-mediated microtubule polymerization.

The identification of an internal repeat within the DCX open reading frame is not surprising, because internal repeats are a hallmark of most well known microtubule-associated proteins, including MAP1B, MAP2C, and tau (15, 19, 27, 28). However, the repeated domain of DCX is quite unique in both its length and number of repeats. DCX has two highly conserved ≈90-amino acid repeats, whereas MAP1B, MAP2C, and tau have between three and twenty-one repeats that vary in length from 4 to 18 amino acids. The composition of the region of the repeats between DCX and other microtubule-associated proteins is similar, however, with a high percentage of basic residues and a basic pI. Notably, the pI for each of the DCX

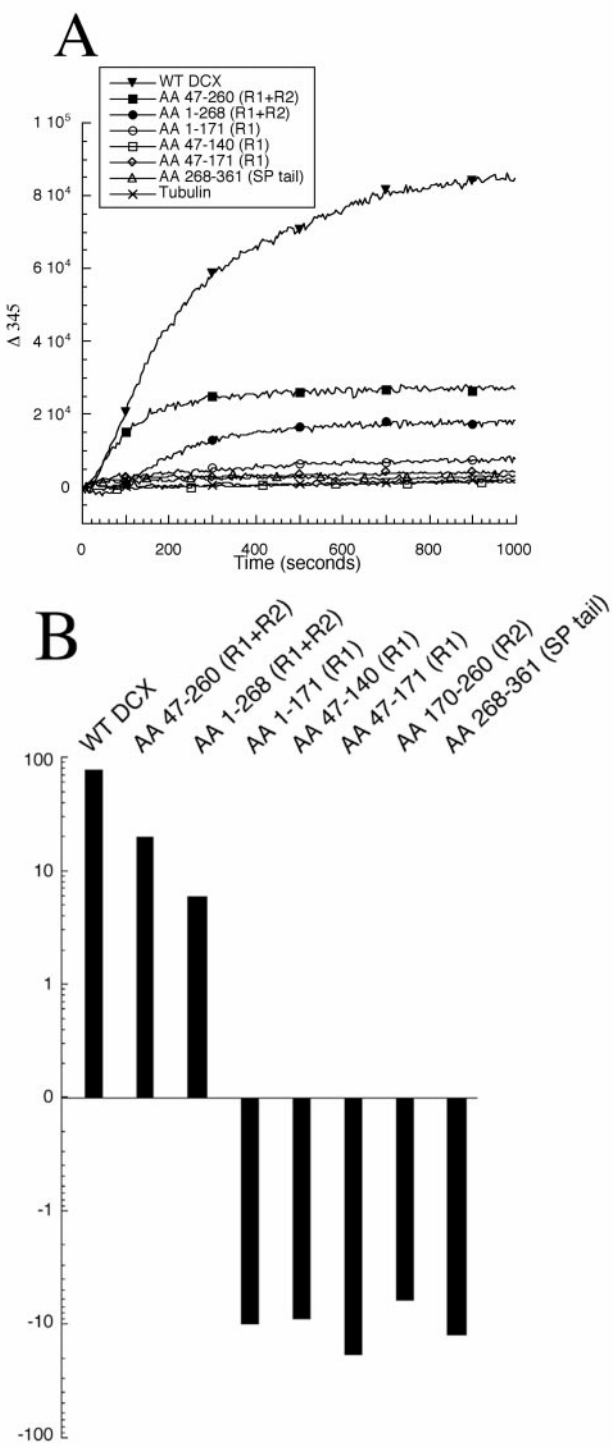

FIG. 6. **Two DCX repeats are sufficient for microtubule polymerization.** *A*, two DCX repeats are sufficient for microtubule polymerization in a turbidity assay. None of the constructs containing a single DCX repeat (AA 1–171, 47–140, and 47–171) nor the serine/proline-rich tail (AA 268–361) polymerized microtubules significantly more than the vehicle control, whereas each of the fragments with two repeats (AA 47–260 and 1–268) led to significant, albeit attenuated, microtubule polymerization, suggesting that two DCX repeats is sufficient for microtubule polymerization. *B*, each of the two-repeat fragments also leads to microtubule polymerization in a pelleting assay. At the completion of the turbidity assay, the mass of the resultant pellet was measured. Each fragment with two repeats led to a significant microtubule pellet, whereas each fragment with one repeat appeared to have an inhibitory effect on microtubule polymerization compared with the vehicle control. (The negative numbers refer to the fact that the pellet from the one-repeat experiments weighed less than the vehicle control pellet.) Results are plotted on a pseudo-logarithmic scale to simplify data interpretation.

repeats is between 9.7 and 9.9, and DCX itself has a pI of approximately 9.3. Therefore, the amino acid residues contained within the DCX repeats are appropriately basic to mediate its microtubule interactions. Interestingly, predicted β-grasp superfolds are not found in other microtubule-associated proteins, based upon analysis using the same algorithms presented above, suggesting that DCX may interact with tubulin in novel ways.

The precise defect of mutant DCX in microtubule polymerization is still unclear. Wild type DCX has been demonstrated to lead to polymerization through an effect on nucleation and bundling of microtubules (6), although there may be a primary effect on polymerization of microtubules independent of nucleation and bundling. Additionally, wild type DCX stabilizes microtubules against depolymerization induced by either exposure to cold or colchicine (5, 6). The results presented here from transfected cells suggest a defect in overall polymerization, but it was not possible to differentiate between microtubule nucleation and bundling based upon the methods used here. Additionally, mutant DCX appears to display a defect in microtubule stabilization against cold depolymerization. It will be interesting to use the DCX patient mutations as a tool to further probe the critical interactions between DCX and microtubules.

If two repeats are all that is required for tubulin polymerization, then what does the rest of the DCX protein do? Some of the naturally occurring *DCX* patient mutations delete just the last 30 amino acids (*i.e.* the carboxyl-terminal region of the serine/proline-rich tail), suggesting that the tail is critical for normal function. This region of DCX may promote protein-protein interactions or somehow positively or negatively regulate the microtubule-interacting effects of DCX. Likewise, the regions surrounding the first repeat appear to positively regulate the ability of the first repeat to co-assemble with microtubules, as the AA 1–171 and 47–171 fragments, containing the 46 amino acids amino-terminal to the first repeat and the inter-repeat region or the inter-repeat region, respectively, partially co-assembled with microtubules. However, the first 46 amino acids and the inter-repeat region are not themselves sufficient for co-assembly with microtubules (data not presented), suggesting that these two regions are not likely to interact directly with microtubules but instead may act as regulators of this binding. Similarly, the AA 1–268 fragment co-assembled with microtubules more efficiently than even the wild type protein, suggesting that the serine/proline-rich tail may function to inhibit the interaction of DCX with microtubules. It will be very interesting to establish the structure of DCX and to identify the physical interactions with tubulin, to better understand how DCX exerts its effects on microtubules.

Although human mutations in several different genes have been shown to lead to an impaired ability of each encoded protein to interact with microtubules, to our knowledge DCX is the first example of patient mutations defining a microtubule-binding domain. For example, Opitz syndrome is a failure of proper closure of midline structures during development in humans, and human mutations in the responsible gene, *midin*, appear to interfere with the ability of the encoded protein to associate with microtubules (29). Similarly, some of the mutations identified in the *neurofibromin* gene, which leads to neurofibromatosis I, impair the ability of the encoded protein to bind to microtubules (30). Perhaps the best known example of mutations in a microtubule-associated protein leading to human disease is tau, wherein mutations lead to frontotemporal dementia and parkinsonism (FTDP-17) (31). Human mutations largely occur around the tau repeats (31), and some of these mutations reduce the ability of tau to bind microtubules and

promote microtubule assembly (32), although there is some controversy regarding this issue (33). The diversity of diseases that are related to impaired microtubule regulation is a reminder of the diversity of capacities and essential roles that microtubules play in nearly all aspects of cellular functioning.

DCX Repeats in Other Novel Proteins Suggest Microtubule Interactions—The presence of two intact DCX repeats in several other human proteins, including the *retinitis pigmentosa 1* (*RP1*) gene, suggest that these two repeats may play essential roles in other biological processes. At least two human proteins, *DCAMKL1* and *RP1*, contain two tandem DCX repeats, and in fact the highest homology between these three proteins is in these repeats. Furthermore, many of the critical amino acid residues as defined by the DCX patient mutations are conserved between all three proteins. Although the function of *DCAMKL1* remains unknown, it appears to bind to and polymerize microtubules,[2] an effect most likely related to the conserved DCX repeats. In addition to a DCX domain in the amino-half of the protein, DCAMKL1 contains a CaM kinase domain in the carboxyl-half (1, 2), suggesting that the DCX domain may be paired with other domains, possibly to mediate specific microtubule-based cellular events. RP1 contains a DCX domain in its amino terminus, although the rest of the 2156-amino acid protein has only minimal similarities to other proteins. All of the identified human mutations in *RP1* are predicted to lead to premature protein termination (34–36), but if the function of the DCX domain in *RP1* is retained, one may predict that human mutations within this domain may be at least partially inactivating.

What Is the Function of DCX in Migrating Neurons?—DCX mutations in humans lead to a defect in cortical neuronal migration (1, 2). Based upon previous data suggesting an effect of DCX on microtubules (5–7), and the present genetic evidence that the patient mutations functionally impair the interaction of DCX with microtubule, DCX likely mediates one or more critical microtubule-based events during neuronal migration. Additionally, DCX is present at sufficient concentrations in neurons to stabilize microtubules, because quantitative Western analysis of neuronally enriched cultures suggest that DCX is present at approximately a 1:70 molar ratio to tubulin,[3] which is within an order of magnitude of the1:15 ratio of DCX:tubulin that leads to optimal DCX-mediated microtubule polymerization *in vitro*.

However, it is still unclear as to which aspects of neuronal migration are defective in neurons with mutant *DCX*. Some humans with lissencephaly display hemideletions in a second gene known as *LIS1* (37), and neurons derived from mice with a targeted hemideletion of this *LIS1* gene display defective neuronal migration (38). It will be very interesting to elucidate the microtubule-based events underlying neuronal migration and determine which aspects of this migration are defective in mice with targeted deletions of these specific neuronal migration genes.

Acknowledgments—We are extremely grateful to members of the Gleeson lab, including E. Christian, C. Lee, E. Leeflang, F. Serneo, T. Tanaka, and M. Lenz for technical and administrative assistance as well as stimulating discussions. We thank P. T. Lin, L. A. Flanagan, S. C. Feinstein, E. Masliah, M. Lu, K. S. Kosik and members of the Walsh lab for helpful discussion and comments. We thank M. M. Blurton-Jones and M. H. Tuszynski for microscopic assistance and S. R. Adams and R. W. Tsien for assistance with fluorimetry.

[2] P. T. Lin, J. G. Gleeson, and C. A. Walsh, unpublished observation.
[3] K. R. Taylor and J. G. Gleeson, unpublished observation

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