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ncd and kinesin motor domains interact with both α- and β-tubulin

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ABSTRACT **Motor domains of the *Drosophila* minus-end-directed microtubule (MT) motor protein ncd, were found to saturate microtubule binding sites at a stoichiometry of approximately one motor domain per tubulin dimer. To determine the tubulin subunit(s) involved in binding to ncd, mixtures of ncd motor domain and MTs were treated with the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide) (EDC). EDC treatment generated covalently cross-linked products of ncd and α-tubulin and of ncd and β-tubulin, indicating that the ncd motor domain interacts with both α- and β-tubulin. When the *Drosophila* kinesin motor domain protein was substituted for the ncd motor domain, cross-linked products of kinesin and α-tubulin and of kinesin and β-tubulin were produced. EDC treatment of mixtures of ncd motor domain and unassembled tubulin dimers or of kinesin motor domain and unassembled tubulin dimers produced the same motor–tubulin products generated in the presence of MTs. These results indicate that kinesin family motors of opposite polarity interact with both tubulin monomers and support a model in which some portion of each protein's motor domain overlaps adjacent α- and β-tubulin subunits.**

Several proteins use microtubules (MTs), polymers of αβ tubulin heterodimers, as "roadways" to perform a variety of essential cellular processes (1, 2). The mechanisms underlying motor activity are poorly understood, in part because interactions of motors with their partner filaments, MTs, are not well understood. A further complication for MT motors is that movement is polar, and therefore, the determinants of directionality must be taken into account. Identification and characterization of the motor–MT interface should help elucidate the processes involved in force production and directional movement.

To date, tubulin-binding sites on motor proteins have been identified only at a coarse level. For members of the kinesin family, a highly conserved region of ≈340 aa forms the head or motor domain (3–5). Within this domain, the region responsible for binding tubulin has been "limited" to 120–170 aa (or ≈20% of the entire kinesin sequence) (3, 4). There is less information concerning the tubulin-binding site on dynein, although some regions have been suggested based on sequence alignment (6). It is not known whether kinesins and dyneins share a conserved tubulin-binding site, each superfamily shares a superfamily-conserved tubulin-binding site, or motors within a superfamily have distinct and different tubulin-binding sites. However, it is clear that despite having similar motor domains, individual members of each superfamily may exhibit distinct biochemical and directional properties. For example, kinesin superfamily proteins may be plus- or minus-end-directed motors (1, 2), and determination of directionality appears intrinsic to the specific motor domain (7).

On the MT side of the interface, several investigations have focused on the C-terminal domains of α- and/or β-tubulin. These 40- to 50-residue domains are exposed on the outer surface of the MT after assembly (8, 9) and are involved in binding MT-associated proteins (10–13) and regulation of assembly (14, 15). However, thus far the data concerning the interaction of these C-terminal domains with MT motors are contradictory. Experiments using modified MTs generated by proteolytic cleavage of the C-terminal regions have produced conflicting results. In one case, removal of only a few residues from α- and β-tubulin appeared to inhibit binding of cytoplasmic dynein to the modified MTs (16), but in another report, binding of kinesin and cytoplasmic dynein to modified MTs was no different from binding to unmodified control MTs (17). A second approach, based on the ability of a polyclonal anti-tubulin antibody to inhibit both flagellar motility and axonal transport, appears to support the involvement of the C-terminal regions of both α- and β-tubulin (18).

From these studies, it is still not clear whether motor proteins bind to α-tubulin and/or β-tubulin, let alone what domains in α- or β-tubulin may interact with MTs. Whether all motors bind the same site(s) on the tubulin dimer is unknown; it has been postulated that kinesin family proteins may bind different sites on tubulin to generate movement in different directions (19, 20).

A more direct approach for demonstrating and characterizing protein–protein interactions, chemical cross-linking, has recently been applied to motor–MT interactions. Used extensively in the actin–myosin system to identify interfacing domains of both actin and myosin (21–23) [and to demonstrate that a tail subunit of axonemal dynein interacts with α-tubulin (24)], cross-linking was employed by Song and Mandelkow (25) to demonstrate that bacterial-expressed kinesin heads could be cross-linked to MTs. Related experiments demonstrated that kinesin heads could also be cross-linked to unassembled tubulin dimers (again via β-tubulin). The cross-linking results provided no evidence for a kinesin interaction with α-tubulin. From these experiments, as well as saturation binding studies (26) and visualization of MTs decorated with kinesin heads (25, 27, 28), it appears that one kinesin head binds one tubulin dimer.

To understand how MT motors generate force and determine polarity of movement, it is important to clearly understand motor–MT binding interactions. Kinesin family members kinesin and ncd share highly conserved motor domains (>40% sequence identity), but move in opposite directions along a MT. To determine whether ncd interacts with MTs in a manner analogous to kinesin, the stoichiometry of ncd binding to MTs was determined and cross-linking experiments were performed on ncd/MT and ncd/tubulin mixtures. Bacterial-expressed ncd heads [MC6 (29)] that exist as monomers and show ATP-dependent MT-binding were used in this study

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Abbreviations: MT, microtubule; TMT, Taxol-stabilized microtubule; EDC, 1-ethyl-3-(3-dimethylaminopropylcarbodiimide); S-NHS, *N*-hydroxysulfosuccinimide; Mg·p[NH]ppA, Mg salt of adenosine 5′-[β,γ-imido]triphosphate.

to avoid possible complications due to formation of internal ncd cross-links. Based on concentration-dependent binding studies, the ncd motor domain binds MTs at a ratio of 1 head per tubulin dimer. By using the cross-linking methods published for kinesin motor domain (25), MC6/MT and MC6/tubulin mixtures were treated with 1-ethyl-3-(3-dimethylaminopropylcarbodiimide (EDC). In both cases, two major products were generated. Immunological analysis with antibodies to tubulin and MC6 indicated that these corresponded to an α-tubulin and MC6 product and a β-tubulin and MC6 product. In addition, similar experiments with a kinesin motor domain protein [DKH340 (26)] different from that used by Song and Mandelkow (25) demonstrated that kinesin heads could also be cross-linked to α- and β-tubulin. The data indicate that kinesin family motors moving in opposite directions interact with both α- and β-tubulin. Based on a binding stoichiometry of one motor domain per tubulin dimer reported here for ncd and elsewhere for kinesin (26), it is probable that kinesin family motors interact with MTs at the intradimer or interdimer junctions between α- and β-tubulin.

METHODS

ncd motor domain protein (MC6) was prepared by the methods of Chandra *et al.* (29). MgATP was present at 0.1 mM throughout the purification process. After elution of MC6 protein from the S-Sepharose column with PB (10 mM sodium phosphate, pH 7.4/1 mM $MgCl_2$/1 mM EGTA/1 mM dithiothreitol) containing 0.2 M NaCl, the protein was quick frozen in liquid nitrogen and stored at −70°C. Approximately 33% of the MC6 protein in these preparations did not bind Taxol-stabilized MTs (TMTs) even in the presence of 2 mM adenosine 5′-[β,γ-imido]triphosphate, Mg salt (Mg·p[NH]ppA). All MC6 protein concentrations given in this report refer to the active fraction that was competent to bind TMTs in the presence of Mg·p[NH]ppA.

The kinesin head protein DKH340 (26) was generously supplied by D. Hackney (Carnegie Mellon University, Pittsburgh). Tubulin was purified from porcine brain by phosphocellulose chromatography (30).

Binding reactions were carried out in AB buffer (20 mM Pipes, pH 6.9/1 mM $MgCl_2$/1 mM EGTA/0.5 mM dithiothreitol) containing 50 mM NaCl, bovine serum albumin (0.1 mg/ml), 2 mM Mg·p[NH]ppA, and 25 μM Taxol. After addition of MC6 protein (0.66–13.25 μM) and TMTs (5 μM tubulin dimer), samples were incubated for 20 min at 22°C. TMTs and bound MC6 were sedimented by centrifugation at 80,000 × g for 10 min in a TLA-100.3 rotor. Identical samples were centrifuged without TMTs to control for nonspecific sedimentation of MC6 protein. Supernatant fractions were carefully removed and diluted with 2× gel sample buffer [2× SB = 125 mM Tris·HCl, pH 6.8/4% (wt/vol) SDS/10% (vol/vol) 2-mercaptoethanol/20% (vol/vol) glycerol] or 5× SB. Pellets were resuspended with 1× SB. Samples were electrophoresed on 7% polyacrylamide gels with known amounts of MC6 as standards. After staining with Coomassie blue R-250 and subsequent destaining, MC6 bands were cut out, and the bound dye was extracted and quantified (26).

In the control samples without TMTs, 3–7% of the MC6 protein sedimented nonspecifically. Greater than 95% of the tubulin dimers pelleted under the conditions used.

To generate motor–TMT complexes, motor protein (MC6 = 7 μM or DKH340 = 10 μM) and TMTs (10 μM tubulin dimer) were mixed and incubated in AB buffer containing 2 mM Mg·p[NH]ppA and 25 μM Taxol for 15 min at 22°C. EDC and *N*-hydroxysulfosuccinimide (S-NHS) were added to 2 and 5 mM, respectively. S-NHS was added to enhance the stability of the intermediate product and thereby improve yield (31). The reaction was terminated after 30 min by the addition of 2× SB. Some cross-linking reactions were performed at 37°C for 15 min; identical results were obtained in these experiments.

To examine motor interactions with unassembled tubulin dimer, motor domain protein (MC6 = 7 μM or DKH340 = 10 μM) was combined with tubulin (10 μM) on ice and incubated for 15 min in the presence of 2 mM Mg·p[NH]ppA without Taxol. EDC and S-NHS were added as above and the mixture was incubated on ice for 1 hr. The reaction was terminated by addition of 2× SB.

Gels were made with "low grade" SDS (Sigma L-5750) to enhance separation of α- and β-tubulin (32). MC6 samples were resolved on 7% polyacrylamide gels; DKH340 samples were resolved on 7 or 8% polyacrylamide gels.

To determine the composition of cross-linked products, proteins were separated by SDS/PAGE and transferred to a nitrocellulose membrane. Nitrocellulose membranes were probed with antibodies to tubulin (polyclonal, ICN), α-tubulin (6B11-1, Sigma), β-tubulin [18D6 (33)], and the conserved kinesin family peptide sequence HIPYR (34). Alkaline phosphatase-labeled secondary antibodies and nitro blue tetrazolium/bromochloroindolyl phosphate were used to detect bound primary antibodies.

RESULTS

To determine the concentration dependence of MC6 protein binding to TMTs, various concentrations of MC6 protein were added to a fixed concentration of TMTs, and the mixture was incubated in the presence of Mg·p[NH]ppA and centrifuged to separate unbound motor (in the supernatant) from TMT-bound motor (in the pellet). Supernatant and pellet fractions were subjected to SDS/PAGE, and Coomassie blue staining was used to determine the concentration of MC6 protein in each fraction. After correction for inactive MC6 protein and MC6 protein that sedimented in the absence of MTs, the concentration of MC6 present in the pellet samples was plotted as a function of the concentration of MC6 present in the supernatant samples (Fig. 1). Final active MC6 concentrations ranged from 0.66 μM to 13.25 μM; the final tubulin dimer concentration was 5 μM. Under these conditions, the binding of MC6 saturated at a stoichiometry of 1.2 MC6 molecules per tubulin heterodimer and had a K_d of 0.7 μM.

EDC cross-linking experiments were initially performed on MC6 protein alone (7 μM), TMTs (10 μM tubulin dimer) with bound MC6 (7 μM), and TMTs alone (10 μM tubulin dimer). The MC6/tubulin dimer ratio was maintained at <1:1 to minimize any nonspecific motor–tubulin interaction. After

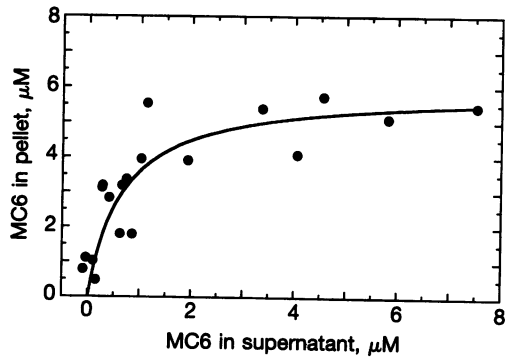

FIG. 1. Concentration dependence of MC6 binding to MTs in the presence of Mg·p[NH]ppA. Taxol-stabilized MTs (5 μM tubulin dimer) were incubated with various concentrations of MC6 and then centrifuged (80,000 × g for 10 min). Concentrations of MC6 in the supernatant and pellet fractions were determined. Each data point is the average of two to four measurements of the bound Coomassie blue dye. Binding stoichiometry and K_d values were determined by fitting the data to a rectangular hyperbola.

termination of the cross-linking reaction, samples were subjected to SDS/PAGE and Coomassie blue staining to assay for the presence of cross-linked product (Fig. 2, lanes 1–3). No defined cross-linked products were observed for EDC-treated MC6 protein in the absence of TMTs (lane 1). In contrast, EDC treatment of MC6-decorated TMTs (lane 2) produced three major products (110, 97, and 92 kDa) and several minor species (>110 kDa). Similar experiments using lower concentrations of MC6 protein (3.5 or 1.75 μM) and the same TMT preparation generated identical products, but at lower yields (data not shown). In comparison, EDC treatment of TMTs (lane 3) generated a single major product (110 kDa) and several minor products (>110 kDa) that did not comigrate with the high molecular mass products generated in the EDC-treated MC6/TMT sample.

A previous study (25) demonstrated that bacterial-expressed kinesin motor domain could be EDC-cross-linked via β-tubulin to unassembled tubulin dimers. To determine whether the MC6 protein could also be covalently cross-linked to free tubulin subunits, the reaction was modified to eliminate MT polymerization. EDC cross-linking was performed on MC6 protein (7 μM), a mixture of tubulin (10 μM dimer) and MC6 (7 μM), and tubulin dimers (10 μM) for 1 hr on ice in the absence of Taxol (Fig. 2, lanes 4–6). As was the case for MC6 protein cross-linked at 22°C in the absence of TMTs (lane 1), no higher molecular mass products were visible for MC6 cross-linked for 1 hr on ice in the absence of tubulin (lane 4). EDC treatment of the MC6/tubulin dimer mixture produced only the 97- and 92-kDa products (lane 5) seen in the MC6/TMT mixture. In addition, the 110-kDa product present in the cross-linked TMTs (lane 3) was not present in the cross-linked tubulin sample (lane 6).

Based on the molecular masses of α-tubulin (55 kDa), β-tubulin (51 kDa), and MC6 (44 kDa) under the conditions used for this SDS/PAGE separation, the size of the major cross-linked products approximated the sum of α-tubulin and β-tubulin (106 kDa), α-tubulin and MC6 (99 kDa), and β-tubulin and MC6 (95 kDa).

Immunological analysis was used to confirm the composition of the three major products identified in Fig. 2. EDC-treated MC6/tubulin and MC6/TMT samples were subjected to SDS/PAGE, transferred to nitrocellulose membrane, and then probed with antibodies to tubulin and to the kinesin family motor domain (Fig. 3). In addition to α- and β-tubulin, a polyclonal anti-tubulin antibody recognized two distinct

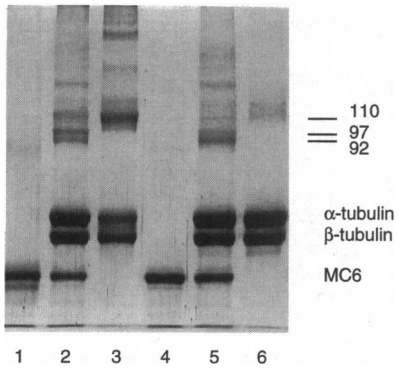

FIG. 2. EDC cross-linking of MC6 to TMTs or unassembled tubulin dimers. Samples were treated with 2 mM EDC and 5 mM S-NHS for 30 min at 22°C (lanes 1–3) or 1 hr at 0°C (lanes 4–6). The products were separated on a 7% polyacrylamide gel and stained with Coomassie blue. Lanes: 1, 7 μM MC6; 2, 7 μM MC6 and TMTs (10 μM tubulin dimer); 3, TMTs (10 μM tubulin dimer); 4, 7 μM MC6; 5, 7 μM MC6 and 10 μM tubulin dimer; 6, 10 μM tubulin dimer. The positions of α-tubulin, β-tubulin, and MC6 are indicated at right. Major cross-linked products present in some of the samples are also indicated at right (in kDa).

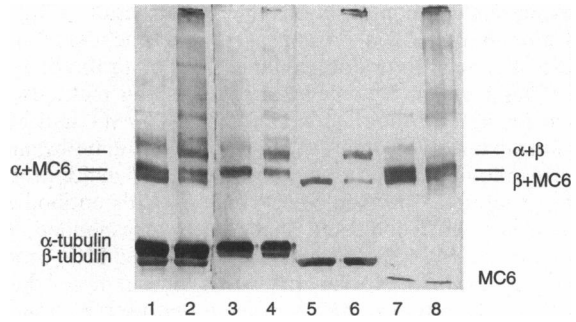

FIG. 3. Immunological analysis of EDC-treated MC6/TMT and MC6/tubulin samples. EDC-treated MC6/tubulin (lanes 1, 3, 5, and 7) and MC6/TMT (lanes 2, 4, 6, and 8) samples were subjected to SDS/PAGE (7% gels) and transferred to nitrocellulose. Nitrocellulose membranes were then probed with antibodies to tubulin (polyclonal; lanes 1 and 2), α-tubulin (6B11-1; lanes 3 and 4), β-tubulin (18D6; lanes 5 and 6), and a conserved kinesin family peptide sequence (HIPYR; lanes 7 and 8). The positions of α-tubulin, β-tubulin, and MC6 are indicated, as are the deduced components of the major cross-linked products.

higher molecular mass products in the MC6/tubulin sample (lane 1) and three in the MC6/TMT sample (lane 2). On the basis of relative intensity and mobility, these products corresponded to the major bands seen in Fig. 2. Of these bands, the 97-kDa band in the MC6/tubulin sample (lane 3) and the 110- and 97-kDa products in the MC6/TMT sample (lane 4) were recognized by an antibody specific for α-tubulin. In comparison, the 92-kDa band in the MC6/tubulin sample (lane 5) and the 110- and 92-kDa products in the MC6/TMT sample (lane 6) were recognized by an antibody specific for β-tubulin. The HIPYR antibody, which recognizes MC6, reacted with the 97- and 92-kDa products, but not the 110-kDa product (lanes 7 and 8). Based on this immunological analysis, the 110-kDa product was composed of covalently bound α- and β-tubulin, the 97-kDa product was composed of α-tubulin and MC6, and the 92-kDa product was composed of β-tubulin and MC6.

The cross-linking results obtained with MC6 were different from results reported for kinesin motor domain cross-linking to TMTs and to tubulin subunits. In that case, Song and Mandelkow (25) found kinesin cross-linked to β-tubulin but

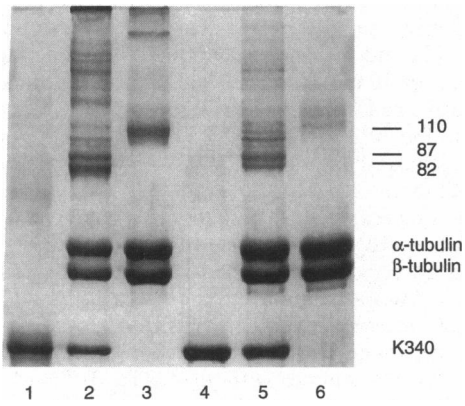

FIG. 4. EDC cross-linking of DKH340 to TMTs or unassembled tubulin dimers. Samples were treated with 2 mM EDC and 5 mM S-NHS for 30 min at 22°C (lanes 1–3) or 1 hr at 0°C (lanes 4–6). The products were separated on a 7% polyacrylamide gel and stained with Coomassie blue. Lanes: 1, 10 μM DKH340; 2, 10 μM DKH340 and TMTs (10 μM tubulin dimer); 3, TMTs (10 μM tubulin dimer); 4, 10 μM DKH340; 5, 10 μM DKH340 and 10 μM tubulin dimer; 6, 10 μM tubulin dimer. The positions of α-tubulin, β-tubulin, and DKH340 are indicated at right. Major cross-linked products present in some of the samples are also indicated at right (in kDa).

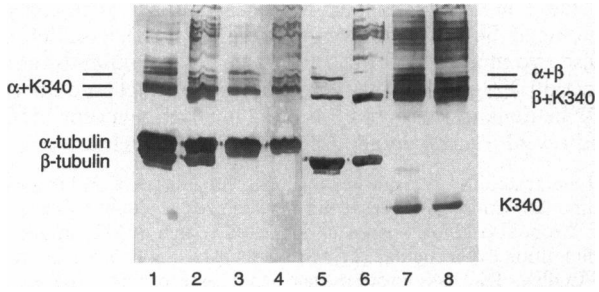

FIG. 5. Immunological analysis of EDC-treated DKH340/TMT and DKH340-tubulin samples. EDC-treated DKH340/TMT (lanes 1, 3, 5, and 7) and DKH340/tubulin (lanes 2, 4, 6, and 8) samples were subjected to SDS/PAGE (8% gels) and transferred to nitrocellulose. Nitrocellulose membranes were then probed with antibodies to tubulin (polyclonal; lanes 1 and 2), α-tubulin (6B11-1; lanes 3 and 4), β-tubulin (18D6; lanes 5 and 6), and a conserved kinesin family peptide sequence (HIPYR; lanes 7 and 8). The positions of α-tubulin, β-tubulin, and DKH340 are indicated, as are the deduced components of the major cross-linked products.

not α-tubulin. Although there may be a number of reasons for the different results (see *Discussion*), in light of the ncd motor domain results, cross-linking experiments were performed in which the kinesin motor domain protein DKH340 (26) was substituted for MC6 (Fig. 4). In these experiments, the ratio of DKH340/tubulin dimer was 1:1 [the approximate saturation point of kinesin head binding to MTs (26)]. EDC treatment of DKH340 produced no significant clearly defined cross-linked products (lanes 1 and 4). As expected from Fig. 2, a major product of 110 kDa was observed in the EDC-treated TMT sample (Fig. 4, lane 3), and no well-defined products were observed in the cross-linked tubulin sample (Fig. 4, lane 6). Two products (87 and 82 kDa) unique to both DKH340/TMT (Fig. 4, lane 2) and DKH340/tubulin (Fig. 4, lane 5) samples were detected. Based on the molecular mass of α-tubulin (53 kDa), β-tubulin (48 kDa), and DKH340 (34 kDa) under the conditions used for this SDS/PAGE separation, the size of the major cross-linked products approximated the sum of α-tubulin and β-tubulin (101 kDa), α-tubulin and DKH340 (87 kDa), and β-tubulin and DKH340 (82 kDa).

To identify the components of the three major cross-linked products observed in Fig. 4, DKH340/TMT and DKH340/tubulin samples were separated and probed with the tubulin and HIPYR antibodies (Fig. 5). As was the case for MC6, in addition to α- and β-tubulin, the polyclonal anti-tubulin recognized three distinct higher molecular mass products in the DKH340/TMT sample (lane 1) and two in the DKH340/tubulin sample (lane 2). On the basis of relative intensity and mobility, these products corresponded to the major bands seen in Fig. 4. Of these bands, the 87-kDa product in the DKH340/tubulin sample (Fig. 5, lane 4) and 110- and 87-kDa products in the DKH340/TMT sample (Fig. 5, lane 3) were recognized by the antibody specific for α-tubulin. In comparison, the 82-kDa product in the DKH340/tubulin sample (Fig. 5, lane 6) and 110- and 82-kDa products in the DKH340/TMT (Fig. 5, lane 5) sample were recognized by the antibody specific for β-tubulin. The HIPYR antibody, which recognizes DKH340, reacted with the ≈87- and 82-kDa products, but not with the 110-kDa product (Fig. 5, lanes 7 and 8). Based on this immunological analysis, the 110-kDa product was composed of covalently bound α- and β-tubulin, the 87-kDa product was composed of α-tubulin and DKH340, and the 82-kDa product was composed of β-tubulin and DKH340.

DISCUSSION

The major finding of this paper is that the motor domains of ncd and kinesin interact with both α- and β-tubulin. The concentration dependence of MC6 binding suggests that one ncd motor domain binds one tubulin heterodimer at saturating MC6 concentrations. This binding stoichiometry is consistent with that reported for a kinesin head domain protein (DKH340) that also exists as a monomer (26). However, previous experiments with DKH340 have also shown that it was possible to achieve binding of more than one DKH340 head protein per tubulin dimer when DKH340 was in excess of tubulin dimers in MTs. It is not clear what contributes to this binding, i.e., whether DKH340 monomers associate with each other or whether there are two motor-binding sites with different affinities on the MT. In the MC6 binding experiments presented here, there was no evidence for the significant interaction of more than one MC6 per tubulin dimer. This result is important because it limits the possibility of artifactual products due to cross-linking of low-affinity or nonspecific interactions.

The kinesin motor domain binds to MTs with an axial periodicity of 8 nm (the length of a tubulin dimer) (25, 27, 28) and appears to step in 8-nm increments from one binding site to the next as it moves along a MT (35). The 1:1 binding stoichiometry reported here for MC6 suggests that ncd, like kinesin, will bind to MTs with an 8-nm axial periodicity and will have a step size of ≈8 nm.

Chemical cross-linking is perhaps the classic method for identification of interacting proteins and this approach was used to identify the tubulin subunit(s) that bind the ncd motor domain. Cross-linking reactions were carried out with the water-soluble zero-length cross-linker EDC. EDC generates covalent bonds between amino and carboxyl groups that interact directly and is, therefore, useful in identifying proteins that closely interact. This specificity is also a potential disadvantage; EDC can directly demonstrate if such an interaction exists but cannot rule out protein interaction by some other mechanism (e.g., hydrophobic interactions).

The finding that MC6 could be covalently linked to α- and β-tubulin and, therefore, closely interacted with both tubulin monomers was somewhat surprising since Song and Mandelkow (25) had found that the kinesin motor domain was linked to β-tubulin alone. One possible explanation for this difference could be that kinesin and ncd contain distinct and different tubulin binding sites and that these sites are involved in the ability of the two motors to move in opposite directions. However, when kinesin motor domain was substituted for MC6 in cross-linking experiments, two major products were produced that corresponded to covalent complexes of kinesin motor domain and α-tubulin and of kinesin motor domain and β-tubulin. Thus, the kinesin motor domain, like the ncd motor domain, closely interacts with both α- and β-tubulin.

As Song and Mandelkow (25) point out, although their results provided no evidence for kinesin interaction with α-tubulin, they could not exclude the possibility due to limitations resulting from the specificity of EDC. There are at least two possible reasons why a kinesin motor domain interaction with α-tubulin may not have been observed. (*i*) Song and Mandelkow (25) used squid kinesin motor domain (as opposed to the *Drosophila* motor domain used here); it is possible that squid kinesin may not present the necessary EDC-reactive groups in sufficient proximity. (*ii*) The antibody to α-tubulin used in that report failed to recognize cross-linked αβ tubulin dimer (as well as higher molecular mass products) that was recognized by the β-tubulin antibody. A possible contributing problem is that gels used in that report may not sufficiently separate α- and β-tubulin bands, so that individual cross-linked products may not be recognized by the anti-kinesin antibody. In the SDS/PAGE procedures used in this paper, replacement of "low grade" SDS with electrophoresis grade SDS caused the two tubulin-motor domain cross-linked products to migrate as a single band.

The finding that ncd and kinesin motor domains interact with both α- and β-tubulin suggests that the motor domains of these proteins probably do not bind completely on top of either tubulin monomer, but rather have some overlap between the

two monomers. A previous EDC cross-linking study (36) on tubulin provided evidence for a model in which the intradimer bond is formed by the C-terminal region of β-tubulin and the N-terminal region of α-tubulin, while the interdimer bond is formed by the C-terminal region of α-tubulin and the N-terminal region of β-tubulin. If these assignments are correct, then two possible sites of α-tubulin–β-tubulin interaction exist for binding ncd and/or kinesin motor domains. The motor head may bind at or near the intradimer junction; this site would give a binding stoichiometry of one motor domain per tubulin dimer and would predict that the motor interacts with the C terminus of β-tubulin and the N terminus of α-tubulin. Alternatively, the motor head may bind at or near the interdimer junction, which would give a binding stoichiometry of approximately one motor domain per tubulin dimer and would predict that the motor interacts with the C terminus of α-tubulin and the N terminus of β-tubulin. The extent of overlap may vary for each motor domain, but the cross-linking results indicate that there is sufficient overlap and that the interaction is close enough to yield EDC-mediated product. In addition, if the Kirchner and Mandelkow model (36) is correct, the stoichiometry and binding periodicity results reported here and elsewhere (25–28) would argue against the possibility that the C-terminal regions of both α- and β-tubulin interact with kinesin family motor domains.

Currently there is no strong evidence favoring the intradimer or the interdimer site. Although it is possible that the ncd motor domain binds at one junction and the kinesin motor domain binds at the other (and, therefore, ncd and kinesin interact with different domains on α- and/or β-tubulin), experiments examining competition between the motors (for MT binding sites) indicate that they bind at overlapping or identical sites on the MT (R. A. Cross, personal communication). Interaction between adjacent monomers within a dimer or between monomers of adjacent dimers is consistent with studies demonstrating that kinesin movement is directed along the long axis of the MT with little lateral displacement and suggests that kinesin moves along one protofilament or perhaps between two adjacent protofilaments (37–39). Although it is conceivable that overlap occurs between α- and β-tubulin subunits on adjacent protofilaments, the B lattice organization of subunits within the MT (25, 40) and the size of the motor domain proteins (smaller than a tubulin monomer) would argue against a motor domain spanning the necessary distance from α-tubulin in one protofilament to β-tubulin in the adjacent protofilament (unless all interactions occur at the seam). Recent structural studies of MTs decorated with kinesin heads do not clearly favor an intradimer or interdimer site but do appear to rule out binding α- and β-tubulin subunits located in adjacent protofilaments (41).

For both motors, the relative yields (intensities) of α-tubulin–motor and β-tubulin–motor products were variable (Figs. 2 and 4), so that it is difficult to draw any conclusions regarding the relative affinities of the motor for the two tubulin monomers. However, one reproducible difference may suggest the site of interaction. EDC-treated TMTs but not EDC-treated tubulin dimers generated cross-linked αβ product, suggesting that cross-linking occurred between α and β subunits of adjacent dimers along a protofilament rather than between the subunits within the dimer. In experiments in which either motor is used to decorate TMTs, the yield of the cross-linked αβ tubulin product was substantially reduced (Figs. 2 and 4, lanes 2 vs. lanes 3). One interpretation is that the presence of the bound motor domain at the junction between dimers protects adjacent tubulin dimers from EDC cross-linking, but this will remain speculative until identification of the specific tubulin domains that interact with motors.

In summary, two kinesin family members with different directional properties, ncd and kinesin, interact with both α- and β-tubulin. Given the stoichiometry of binding of both protein's motor domains to MTs (26) and the periodicity of binding of the kinesin motor domain (25, 27, 28), it is likely that these two motors bind MTs at or near an α-tubulin–β-tubulin junction. Fragmentation of cross-linked products should identify the domains of α- and β-tubulin involved in motor-binding and reveal a more detailed picture of the interface.

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