Structural and Functional Domains of the *Drosophila* ncd Microtubule Motor Protein*

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Nonclaret disjunctional (ncd) is a kinesin-related microtubule motor protein that is required for proper chromosome distribution in *Drosophila*. Despite its sequence similarity to kinesin heavy chain, ncd translocates with the opposite polarity as kinesin, toward microtubule minus ends. We have expressed different regions of the protein in bacteria and analyzed the proteins for function. Results indicate that ncd consists of three domains: a basic, proline-rich N-terminal "tail," a central α-helical coiled-coil stalk, and a C-terminal motor domain. The ncd N terminus proteins bundle microtubules in motility assays and show ATP-independent binding to microtubules in solution. Truncated proteins, lacking the tail but containing the predicted motor domain and differing lengths of the stalk, did not support microtubule gliding in *in vitro* assays but showed microtubule-stimulated MgATPase activity in solution. Addition of a nonspecific N terminus to two of the truncated proteins restored directional gliding and rotation of microtubules in motility assays, demonstrating that these properties map to the predicted mechaanochemical domain of ncd. Physical properties of the C terminus proteins indicate that the stalk region is important for dimerization and that the Ncd protein probably exists as a dimer.

Nonclaret disjunctional (ncd) is a microtubule motor protein that is required in *Drosophila* for proper distribution of chromosomes in meiosis and early mitosis (Sturtevant, 1929; Lewis and Gencarella, 1952; Davis, 1969; Yamamoto et al., 1989; Endow et al., 1990). ncd has been localized to spindles of *Drosophila* oocytes and early embryos using antibodies directed against nonconserved regions of the protein (Hatsumi and Endow, 1992b). Association with the spindle, together with the abnormal spindles observed in mutant oocytes (Wald, 1996; Kimble and Church, 1983; Hatsumi and Endow, 1992a) and embryos (Hatsumi and Endow, 1992a), suggests that ncd functions as a spindle motor in meiosis and early mitosis. The motor may play an essential role in spindle assembly and spindle pole formation in meiosis (Hatsumi and Endow, 1992b).

Bacterially expressed ncd protein is a microtubule motor that translocates toward the minus ends of microtubules, in the opposite direction as kinesin, with a velocity of 4-15 μm/min (Walker et al., 1990; McDonald et al., 1990). Microtubules rotate as they glide on ncd-coated glass surfaces, indicating that the protein generates torque as it moves on microtubules (Walker et al., 1990). The ncd motor protein also cross-links and bundles microtubules (McDonald et al., 1990).

The predicted full-length ncd protein is 700 residues starting from the first AUG and has a Ms of 77,500 (Endow et al., 1990). Three domains can be identified based on predictions from the primary amino acid sequence and sequences conserved among the kinesin family of proteins. The N-terminal "tail" region consists of ~200 amino acids that are extremely basic (pI = 12.2) and proline-rich (10.4%). The central region of the protein (residues ~199-355) consists of heptad repeats of hydrophobic amino acids, many of which are leucines at the "d" position, that, in an α-helix, are characteristic of coiled-coil interactions (Landschulz et al., 1988; Turner and Tjian, 1989; O'Shea et al., 1991; Hodges, 1992). This region may form a structure similar to the "stalk" of kinesin heavy chain (Hirokawa et al., 1989; de Cuevas et al., 1992). The predicted motor domain of ncd is globular in structure and is at the C terminus of the protein (residues ~356-700), in contrast to the N-terminal location in kinesin. The C terminus contains a region of ~320 residues that are 41% identical in sequence to the mechaanochemical domain of kinesin heavy chain (Endow et al., 1990; McDonald and Goldstein, 1990).

During the past 2 to 3 years, several proteins (KAR3, bimC, cut7, ncd, unc-104, Eg5) with sequence similarity to kinesin have been identified (reviewed in Endow and Titus, 1992). These proteins contain a region similar to the motor domain of kinesin, but differ in the remainder of the molecules from kinesin heavy chain and from each other. Although several kinesin-related proteins have been identified, only ncd, Eg5 (Sawin et al., 1992), and MKLP-1 (formerly CHO1; Nislow et al., 1992) have been demonstrated to function as microtubule motors *in vitro*. Like kinesin, Eg5 and MKLP-1 are plus-end microtubule motors. ncd is the only minus-end-directed kinesin motor protein identified thus far. The striking differences in the overall domain organization and cellular functions of ncd and kinesin heavy chain suggest that other properties of the proteins may also differ.

As a first step to understanding how the ncd protein functions, we have constructed plasmids that express only the N-terminal tail of the protein (N terminus proteins) or the C-terminal motor domain with different lengths of the stalk (C terminus proteins). The purified bacterially expressed proteins have been analyzed for functional characteristics, and their physical properties have been determined. The results of these studies, together with rotary-shadowing electron mi-

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crosscopy of two of the proteins, allow assignment of functional and structural domains to the ncd protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

5'-43P-5'P-TPATP, 5'-43P-dATP, and 5'-43P-dGTP were purchased from Amersham; leupeptin, pepstatin A, aprotinin, N-p-tosyl-L-arginine methyl ester, ATP, AMP-PNP, DTT, and EGTA were from Sigma; Norit A activated carbon was from Aldrich; restriction endonucleases T4 DNA ligase, mung bean nuclease, DNA ligase, and the Klenow fragment of DNA polymerase I were from New England Biolabs, Inc.; and isopropyl β-d-thiogalactopyranoside was from United States Biochemical Corp. Tubulin was purified from porcine brain (Walker et al., 1988), and taxol was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

**Methods**

Construction of Plasmids for Protein Expression

pET/N1—A DNA fragment encoding the first 324 amino acids of the predicted 700-residue ncd protein (Endow et al., 1990) was synthesized by PCR from a 0-4-h Drosophila embryo cDNA library (Brown and Kafatos, 1988) using the forward and reverse primers, 5'-TGC ATT CTT TAT TTA TCG-3', respectively. The gel-purified PCR fragment was ligated to Ndel-digested and repaired pET 3b (Studier et al., 1990). The sequence and reading frame of the insertion were determined by DNA sequence analysis using the dideoxy chain termination method (Sanger et al., 1977). The sequence analysis revealed a 2-base pair deletion at residue 183. This was repaired by replacing the EcoRI-AflII fragment containing the deletion with the corresponding fragment from a wild type cDNA clone. The N1 protein is expressed as a nonfusion protein.

pET/MC5—A DNA fragment encoding amino acids 1-204 of the 700-residue ncd protein was synthesized by PCR as for pET/N1, using the same forward primer and the reverse primer, 5'-TGA CTT TGT CTT AAG CAC C3' 5'. The gel-purified PCR product was ligated to the repaired Ndel site of pET 3b. DNA sequence analysis revealed a 2-base pair deletion in the insertion at residue 205, resulting in a protein with the first 204 amino acids of ncd followed by 4 novel amino acids (ANGA) and a stop codon. The protein is expressed as a nonfusion protein.

pET/MC5—MC1 was constructed from the previously described pET/ncd plasmid (Walker et al., 1990) as described in Chandra et al. (1993). DNA sequence analysis of the construct was carried out to confirm that the insertion was unaltered and in-frame. The protein expressed from the pET/MC1 plasmid contains 11 residues of bacteriophage T7 T10 protein, 2 linker amino acids, and 492 residues of ncd, corresponding to amino acids 209-700.

pET/MC5—MC5 was constructed by excising a 1.5-kb DNA fragment from the previously described pET/ncd plasmid by digestion with Sphl, followed by incubation with the Klenow fragment of DNA polymerase I, then digestion with EcoRI. The 1.7-kb ncd fragment was gel-purified and ligated to gel-purified pET 3b that had been digested with BamHI, repaired, and then restricted with EcoRI. The MC5 protein was expressed as a nonfusion protein.

**Expression and Purification of ncd Proteins**

pET plasmid constructs were transformed either into BL21(DE3) or BL21(DE3)LyS3 host cells carrying the bacteriophage T7 RNA polymerase gene under the control of the isopropyl β-d-thiogalactopyranoside-inducible lac UV5 promoter (Studier et al., 1990). pGEX plasmid recombinants were transformed into BL21 cells for protein expression.

Cultures were grown and induced, and lysates were made as described in Chandra et al. (1993) and Chandra and Endow (1992). Briefly, cells were grown at 37°C to A660 = 0.5-0.7 and induced by addition of isopropyl β-d-thiogalactopyranoside to 0.2-0.4 mM and rapid shacking for 4-5 h at 24°C. Packed induced cells were suspended in 3.5 ml/g wet weight of AB (20 mM PIPES, pH 6.9, 1 mM MgCl2, 1 mM EDTA, 0.5 mM DTT) + 0.1 mg/ml lysozyme + protease inhibitors and incubated for 30 min on ice. Following 2 cycles of freeze/thaw, lysates were made 40 μg/ml DNase I + 10 mM MgCl2, kept for 10 min on ice, and then centrifuged at 27,000 X g for 20 min.

The N1 protein was purified from the 27,000 X g pellet by washing the pellet in AB containing 0.4% Triton X-100, followed by centrifugation at 27,000 X g for 15 min. The low top pellet was solubilized in 5 ml of 6 M urea + 10 mM glycine in PB (10 mM NaPO4, pH 7.4, 1 mM MgCl2, 1 mM EDTA, 0.5 mM DTT) + 0.1 mg/ml lysozyme + protease inhibitors and incubated for 4 h on ice, and then centrifuged at 105,000 X g for 10 min to remove insoluble material. The supernatant was kept on ice overnight, then loaded onto a 1-mL S-Sepharose (Pharmacia Biotechnology Inc.) column. After washing with 6 M urea + 10 mM glycine in PB, the column was eluted with 0.1 M NaCl in urea + glycine in PB. The N1 protein was eluted at 0.2 M NaCl and was dialyzed against 5% sucrose in PB overnight at 4°C and stored frozen at -20°C.

The N2 protein was purified from the insoluble fraction of induced cells as described in Hatsumi and Endow (1992b) by solubilizing a 29,000 X g pellet from a sucrose cushion in 3.5 M guanidine HCl. After dilution of the guanidine HCl solution into PB, the precipitated protein was recovered by centrifugation and extracted with 20 mM MOPS, pH 5, followed by 20 mM MOPS, pH 8 ± 0.3 M NaCl. Protein in the MOPS + NaCl extraction was fractionated on an ACA 44 gel filtration column, and peak fractions were concentrated and stored at ~70°C. Purification of the N1 and N2 proteins was monitored by cross-reactivity on Western blots with an antibody directed against the terminal 182 amino acid residues of ncd (Hatsumi and Endow, 1992b).

The C terminus proteins (MC1, MC5, MC6, and the corresponding GST fusion proteins) were purified from the 27,000 X g cell lysate supernatant, as described for MC1 in Chandra et al. (1991). Briefly, protein was eluted from a 1-mL Sepharose column in PB with steps of 0.1-0.5 M NaCl in PB. The C terminus proteins eluted at 0.1-0.2 M NaCl. The purified proteins were dialyzed against PB and stored frozen at ~70°C. Purification of the proteins was monitored by cross-reactivity on immunoblots with an antibody (a gift of K. Sawin) directed against the conserved HIPYRESKLT motif in the motor domain of the kinesin-related proteins (Endow, 1991).

Analysis of Motility Data

Microtubule velocities were measured as described previously (Walker et al., 1988) using a mouse-driven video cursor overlaid on images played back from videotapes. Home-written software was used for

1. The abbreviations used are: AMP-PNP, adenosine 5'-beta,gamma-imino triphosphate; DTT, dithiothreitol; PCR, polymerase chain reaction; kb, kilobase(s); PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; GST, glutathione S-transferase.
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to display positions of microtubule ends with time and to calculate displacement/min. Velocities were determined by measurement of >10 microtubules for three of the proteins (near full-length ncd, GST/MCl, GST/MC5). For GST/MC6, most of the microtubules did not move, and velocities were obtained for only 5 microtubules.

Physical Properties of the C Terminus Proteins

Sedimentation coefficients (s20,w) were estimated by rate zonal sedimentation of purified proteins on 5-20% linear sucrose gradients (Martin and Ames, 1961) in PB containing 0.2 M NaCl. Protein standards (cytochrome c, 1.75 S; bovine serum albumin, 4.6 S; rabbit muscle creatine kinase, 5.3 S; rabbit muscle aldolase, 7.3 S) were centrifuged in a separate gradient at the same time. The sedimentation distance was linear with s value over this range.

The Stokes radius (Rs) was determined by fast protein liquid chromatography on a Superose 12 column (Pharmacia Biotechnology Inc.) equilibrated with PB containing 0.2 M NaCl and 0.02% Tween 20. The column was calibrated using proteins of known Stokes radii: bovine blood fibrinogen (8.2 nm), horse spleen ferritin (6.1 nm), rabbit muscle aldolase (4.8 nm), bovine serum albumin (3.5 nm), rabbit muscle creatine kinase (3.62 nm), and horse heart cytochrome c (1.2 nm).

RESULTS

Expression and Purification of Recombinant Proteins—In order to identify the structural and functional domains of the ncd protein, we have constructed plasmids that express different regions of the protein. Fig. 1 illustrates the proteins used in these studies together with full-length ncd and the near full-length ncd construct described previously (pET/ncd; Walker et al., 1990).

Two proteins, N1 and N2, correspond to the N-terminal tail of ncd, which is not conserved among the kinesin proteins. Three C terminus proteins, MC1, MC5, and MC6, contain the highly conserved, predicted mechanochromical domain at the C terminus of ncd joined to different lengths of the central stalk region. The three C terminus proteins were also expressed as fusion proteins with glutathione S-transferase at the N terminus. The bacterially expressed proteins were purified and analyzed for function using in vitro motility, microtubule binding, and ATPase assays.

The N1 and N2 proteins were present in insoluble form when expressed in bacteria and were purified by denaturing the inclusion body pellet in 6 M urea (N1) or 3.5 M guanidine HCl (N2) followed by ion exchange or gel filtration chromatography, respectively. Proteins expressed from the C terminus constructs showed much greater solubility and were purified by passing the bacterial lysate over an S-Sepharose ion exchange column. Most of the bacterial proteins did not bind to the column and flowed through, while the C terminus proteins bound and could be eluted with 0.1-0.2 M NaCl. Proteins eluted with >0.3 M NaCl did not support microtubule gliding in motility assays, suggesting that >0.3 M NaCl destabilizes or denatures the proteins. The proteins eluted from the S-Sepharose column were ~90-95% pure. Fig. 2 shows a Coomassie Blue-stained gel of the purified N1 and N2 proteins and the C terminus proteins after elution from an S-Sepharose column. Cross-reaction on a corresponding immunoblot with an anti-peptide antibody directed against the HIPYRESKLT motif, which is conserved among the kinesin proteins, confirmed the correct expression of the C terminal proteins (Fig. 2).

The GST/MC1 protein did not bind microtubules to the coverslip in in vitro motility assays after elution with 0.1 M NaCl from an S-Sepharose column. The cell lysate was therefore precipitated with ammonium sulfate at 40% of saturation, and the resolubilized protein was dialyzed and used in motility assays (Fig. 2, lane 10).

Microtubule Motility Assays—The ability of the bacterially expressed proteins to support microtubule gliding on protein-coated glass surfaces was determined using in vitro motility assays and VE-DIC microscopy (Walker et al., 1988). The N1 and N2 tail proteins did not support microtubule gliding, but caused extensive bundling and binding of microtubules on the glass coverslip. This demonstrates that the basic N-terminal 204 residues of ncd can bind to and bundle microtubules in vitro. Results of motility assays are summarized in Table I.

Microtubule binding and bundling to MC1 will be reported separately (Chandra et al., 1993). The results of this analysis showed that microtubules bound to MC1 on glass exhibit random, one-dimensional diffusion similar to that reported for the β-1C axonemal dynein, which has been interpreted to represent a weak binding state of the motor (Vale et al., 1989).

The MC5 protein consists of ~61 amino acids of the heptad repeat region together with the highly conserved C terminus. Unexpectedly, microtubules bound to MC1 on glass surfaces did not exhibit unidirectional translocation, but instead moved erratically back and forth along their longitudinal axis. A detailed analysis of the movement of microtubules bound to MC1 will be reported separately (Chandra et al., 1993). The MC5 protein contains ~23 residues of the heptad repeat region joined to the conserved C terminus of ncd. Neither MC5 nor MC6 supported microtubule gliding in in vitro motility assays. However, the proteins bound microtubules to the glass surface, and the bound microtubules showed low extents of bundling. The MC5 and MC6 proteins are thus capable of binding microtubules, but do not support microtubule translocation when bound to glass.
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The kinesin proteins; this motif corresponds to residues 619-628 in the C terminus of ncd.

Proteins for motility assays were either purified over an S-Sepharose column (see "Experimental Procedures") or precipitated with ammonium sulfate at 40% of saturation from the 27,000 × g supernatant (GST/MCl), followed by dialysis against PB. Motility assays were performed as described previously (Walker et al., 1990; Chandra et al., 1993) by sequential mixing of 5 μl of protein in PB, 1.6 μl of taxol-stabilized microtubules (tubulin lysate after a 4-hour induction at 24°C; lanes 4-9 show S-Sepharose-purified proteins. Lane 4, MC1 protein; lane 5, MC5 protein; lane 6, MC6 protein; lane 7, GST/MCl protein; lane 8, GST/MC5 protein; lane 9, GST/MC6 protein. Lane 10 shows a 40% ammonium sulfate-precipitated GST/MCl protein used for motility assays. The position of the molecular weight markers is shown on the left. The near full-length ncd protein appears to be migrating abnormally as a 110-kDa protein instead of its actual size of 78 kDa in this gel system. The N1 and N2 proteins do not cross-react with the anti-HIPYRESKLT antibody (lanes 2 and 3 on immunoblot).

**TABLE I**

Motility characteristics of the C terminus proteins

| Protein | Motility | Velocity | Rotation |
|---------|----------|----------|----------|
| ncd     | Minus-end| 8-10     | +        |
| MC1     | Diffusional|         |          |
| MC5     |           |          |          |
| MC6     |           |          |          |
| GST/MCl | Minus-end| 7-10     | +        |
| GST/MC5 | Minus-end| 5-6      | +        |
| GST/MC6 | (+)      | <0.2     | ND*      |

* ND, not determined.

The MC1, MC5, and MC6 proteins were also expressed as fusion proteins with a glutathione-S-transferase protein at the N terminus. The GST/MCl protein supported microtubule translocation at a velocity of 7-10 μm/min, and axone/microtubule complexes rotated as they glided with plus ends leading on protein-coated surfaces. These results indicate that the GST/MCl protein, like near full-length ncd (Walker et al., 1990), moves toward the minus ends of microtubules and generates torque as it moves. The GST/MC5 protein translocated microtubules at a velocity of 5-6 μm/min, and axone/microtubule complexes rotated as they glided with plus ends leading on protein-coated surfaces. As was observed for MC1, addition of a nonspecific protein to the N terminus of MC5 restored the ability of the protein to support directional gliding and rotation of microtubules on glass.

The GST/MC6 protein is only 39 amino acids shorter in length than GST/MC5, but showed little microtubule translocating ability. Microtubules bound efficiently to GST/MC6-coated glass coverslips, but most were stationary. Of eight preparations, microtubule translocation was only observed in one preparation. In this preparation, a few microtubules moved very slowly at a rate of ~0.1-0.2 μm/min. The movement of these microtubules was unidirectional and was measured over a period of 5-10 min. The movement observed indicates that GST/MC6 is capable of microtubule translocation. The polarity of microtubule movement was not determined.

**Microtubule Binding Assays**—The ability of truncated ncd proteins to bind microtubules in solution was determined using microtubule binding/pelleting assays. The purified proteins were incubated at room temperature with taxol-stabilized microtubules, the mixes were centrifuged, and microtubule pellets and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis. Binding of protein to microtubules was measured under three different conditions: 1) absence of 5 mM MgATP, 2) presence of 5 mM MgATP, and 3) presence of 5 mM AMP-PNP. The release of protein from the microtubule + protein pellets upon addition of 10
mm MgATP was determined for pellets formed in the absence of added MgATP or presence of AMP-PNP. Controls of protein + MgATP alone and microtubules + MgATP alone were carried out for each protein tested. The results of these assays are summarized in Table II.

The N1 and N2 tail proteins bind microtubules both in the presence and absence of 5 mM MgATP. Protein bound to microtubules in the absence of MgATP or presence of AMP-PNP could not be released from the microtubule pellets on addition of MgATP (Table I). The N terminus proteins therefore bind strongly to microtubules in these assays in an ATP-insensitive manner.

The C terminus proteins and the corresponding GST forms of the proteins were also tested for binding to microtubules in solution. The proteins bound microtubules both in the absence of ATP and the presence of 5 mM AMP-PNP. Release of the proteins from the microtubule pellet was determined upon addition of 10 mM MgATP. A significant amount of each of the proteins remained associated with the microtubule pellet. The inability of added ATP to release protein bound to microtubules in the absence of ATP or presence of AMP-PNP suggests the presence of an ATP-insensitive microtubule binding site in the C terminus proteins. In contrast to the other C terminus proteins, addition of MgATP released ~80% of GST/MC1 protein that pelleted with microtubules in the presence of AMP-PNP. Exposure of GST/MC1 to 0.1 M NaCl during purification may have altered its interaction with microtubules, resulting in a weaker binding of the motor to microtubules. This is consistent with the observation that the S-Sepharose-purified GST/MC1 used in these assays did not bind microtubules to the glass coverslip in in vitro motility assays.

The majority of the C terminus proteins did not bind microtubules in the presence of 5 mM MgATP. Only GST/MC6 protein pelleted with microtubules in the presence of MgATP.

**ATPase Assays**—The ability of the C terminus proteins and the corresponding GST fusion proteins to hydrolyze ATP was determined in the presence and absence of microtubules using modifications of previously published methods (Chandra et al., 1993; Chandra and Endow, 1992). Results of these assays are shown in Table III. The C terminus proteins showed basal levels of MgATPase activity in the absence of microtubules that ranged from $k_\text{cat} \sim 0.03$–0.15 s$^{-1}$. The value of $k_\text{cat} = 0.15$ s$^{-1}$ for GST/MC1 deviated the greatest from the others and may reflect the greater instability of the protein. In the presence of microtubules, the proteins showed $k_\text{cat}$ values of ~0.2–3.8 s$^{-1}$, indicating 2- to 95-fold stimulation over the basal levels. The C terminus proteins, including the GST fusion proteins, are therefore microtubule-stimulated MgATPases. The stimulation of basal MgATPase activity was greatest for MC6 and GST/MC6, which exhibited $k_\text{cat}$ values that are ≥3 times higher than those of the other proteins.

**Association State of the C Terminus Proteins—Sedimentation Coefficients and Stokes Radii** were determined for the C terminus proteins (Chandra et al., 1993; Chandra and Endow, 1992).

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### Table II

**Binding to microtubules by ncd truncated proteins**

Microtubule binding assays were performed as described in Chandra et al. (1993) by incubating 50–75 μg/ml protein with taxol-stabilized microtubules (tubulin concentration = 0.5 mg/ml) in AB + 0.1 mM MgGTP + 10 μM taxol in a volume of 100 μl. 5 mM MgATP or AMP-PNP was added to some reactions. Control reactions contained protein + MgATP or microtubules + MgATP. Following a 30-min incubation at 24 °C, mixes were centrifuged 20 min at 100,000 $\times g$ at 22 °C. 20 μl of 5 × sample buffer were added to the supernatant and 120 μl of 1 × sample buffer to the pellet, and equal volumes of the pellet and supernatant were analyzed by SDS-PAGE on a 3% stacking and 10% separating gel using the method of Laemmli (1970).

The release of protein from the microtubule + protein and microtubule + protein + AMP-PNP pellets on addition of MgATP was determined by washing the pellet with buffer, resuspending it in 100 μl of 10 mM MgATP for 20 min and recentrifuging the mix. The first three columns show the relative amount of protein present in the microtubule pellets. The release of protein with 10 mM ATP is shown in the last two columns and represents the relative amount of protein in the supernatant. The relative amount of protein in the pellets and supernatants was determined from densitometer scans of wet gels. Values were corrected for protein that pelleted in the absence of microtubules and for the relative width of the gel lanes.

| Protein | + MT | + MgATP | + AMP-PNP | MT | + AMP-PNP |
|---------|------|---------|-----------|----|-----------|
| N1      | ++++ | ++++    | ++++      | -  | -         |
| N2      | ++++ | ++++    | ++++      | -  | -         |
| MC1     | ++   | ++++    | ++++      | -  | -         |
| GST/MC1 | +    | ++++    | ++++      | -  | -         |
| GST/MC5 | ++++ | ++++    | ++++      | -  | -         |
| MC6     | ++++ | ++++    | ++++      | -  | -         |

Table II

### Table III

**Mg$^{2+}$ATPase activity of ncd C terminus proteins**

ATPase assays were performed at 24 °C using modifications of published methods (Cohn et al., 1987; Wagner et al., 1989), as described in Chandra et al. (1993) and Chandra and Endow (1992). Protein concentration was determined using the Bradford colorimetric assay. 300-μl reaction mixtures contained 20 μg/ml protein, 4 mM Mg$^{2+}$, 10 μM taxol + 1 mM MgATP + 1 mg/ml taxol-stabilized microtubules in 15 mM imidazole, pH 7.0, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT. 2 μl of 4 μl of the reaction mix were counted in scintillation fluid to determine the specific activity. 40-μl aliquots were withdrawn at 0, 2, 5, 10, 20, and 40 min into microcentrifuge tubes containing 0.76 ml of 5% activated charcoal suspended in 50 mM NaH$_2$PO$_4$ (Hagashijima et al., 1987). The charcoal mixtures were kept on ice for 15 min, and the tubes were centrifuged at 15,600 $\times g$ for 5 min. The supernatant was run to remove all charcoal particles. Aliquots (150–400 μl) of the final supernatant were taken for scintillation counting. The activated charcoal binds unhydrolyzed ATP but not released Pi, which remains in the supernatant. <2% of the total counts were present as Pi in the 0-min time point. Values obtained for the 2-, 5-, and 10-min time points from 2-3 experiments were pooled and analyzed by linear regression through the origin. Data for MC5 + MT are from one experiment only. The data for MC1 were taken from Chandra et al. (1993). Values were rounded off to the nearest 0.01 (~MT) or 0.1 (+MT). $k_\text{cat}$ = microtubules.

| Protein | $k_\text{cat}$ | Fold stimulation |
|---------|---------------|------------------|
| MC1     | 0.05          | 8                |
| GST/MC1 | 0.15          | 3                |
| MC5     | 0.06          | 3.5              |
| GST/MC5 | 0.03          | 5                |
| MC6     | 0.04          | 95               |

* The S-Sepharose-purified GST/MC1 protein used in these assays did not support microtubule gliding in in vitro motility assays.
terminus proteins and used in calculations of protein molecular weight. The calculated molecular weight was compared with the monomer molecular weight predicted from the amino acid sequence. The data are summarized in Table IV. The ratios of the calculated and predicted monomer molecular weights indicate that MC1, GST/MC1, and MC6 are dimers in solution, while MC6 exists as a monomer. The calculated molecular weight for GST/MC5 suggests that the bacterially expressed protein exists as a mixture of dimers and tetramers. Calculation of the frictional ratios ($f/fo$) indicates that MC1 and GST/MC1 are asymmetric in shape, while MC6 is almost completely globular (Table IV).

**Structure of MC1 and GST/MC1—** Rotary-shadowed images of MC1 protein samples showed several structures in areas with well-spread molecules of good contrast. Approximately 70% of the particles appeared globular with no visible tail, while ~20% were globular with a discernable stalk, as shown in the first two columns of Fig. 3A. A small proportion of the molecules appeared to be paired, as shown in the third column. Measurement of molecules with distinct tails gave dimensions of 6.8 × 6.0 nm for the globular head and 18 nm for the length of the tail from the point it attaches to the globular head. Based on data obtained from hydrodynamic analysis (sedimentation coefficients and Stokes radii), the molecules observed represent parallel dimers with the tail formed by an α-helical coiled-coil and the globular region comprising the two motor domains. The size of the globular region is somewhat smaller than that reported for the dimeric motor domains of kinesin (Hirokawa et al., 1989; Hisanaga et al., 1988), and in our micrographs the two domains are not clearly resolved. The length of the MC1 tail corresponds to approximately 120 residues at a spacing of 0.15 nm per residue in an α-helix. The actual number of residues estimated to be in the α-helix is 147, corresponding to amino acid 209 at the N terminus of MC1 to residue 355 near the N terminus of MC6. The apparent tetramers shown in the third column of Fig. 3A might reflect a tendency of the molecules to form larger oligomers, but this was a small fraction of the molecules observed.

Rotary-shadowed images of GST/MC1 (Fig. 3B) revealed a dumbbell-shaped molecule with two globular regions joined by a short stalk. The two globular domains are slightly different in size, measuring 8.8 nm and 7.2 nm across the largest dimension perpendicular to the axis of the dumbbell. We believe that one of these corresponds to the two motor domains of ncd, while the other represents the two GST domains. The total length of the molecule measured from the outside of the globular domains is approximately 22 nm. This is similar to the length of MC1 and is therefore 4–6 nm shorter than expected for the addition of a globular GST domain to the MC1 protein. This difference implies that the coiled-coil rod of ncd has somehow been shortened by the addition of the GST domains. One possibility is that the initial turns of the α-helix lie along the GST surface, and the molecule therefore appears shorter in the micrographs.

**DISCUSSION**

In order to map the molecular determinants of ncd motor function, we have constructed plasmids for expression of proteins that correspond to different regions of the full-length protein. The truncated ncd proteins were tested for microtubule translocating activity in motility assays and binding to microtubules and ATPase activity in solution. Table V summarizes the results of the motility, microtubule binding, and ATPase assays.

The N terminus tail proteins showed ATP-independent binding to microtubules both in *in vitro* motility assays and solution binding assays. The ability to bind and cross-link microtubules is likely to be important to the *in vivo* function of the ncd protein. In particular, the proposed role of the motor in spindle pole formation has been attributed to the ability of the ncd protein to cross-link microtubules as it moves toward microtubule minus ends, focusing microtubule ends (Hataami and Endow, 1992a, 1992b). Similar microtubule binding regions in the non-motor domains of the proteins have been reported for Kar3 (Meluh and Rose, 1990) and human kinesin heavy chain (Navone et al., 1992), based on studies using transformed or transfected cells. The microtubule binding region in the N terminus of Kar3 is likely to correspond to the basic, proline-rich region that is similar with respect to these properties to the N terminus of ncd. In ncd, the interactions of the N-terminal tail with microtubules may be regulated *in vivo* by association with light chains or other proteins.

The C terminus proteins (MC1, MC5, MC6) do not support gliding of microtubules on protein-coated glass, but retain the ability to hydrolyze MgATP in solution. Addition of a non-specific N terminus, a 26-kDa GST protein, to two of the C terminus proteins (MC1 and MC5) restored the ability of the proteins to support directional gliding and rotation of microtubules.

**TABLE IV**

| Physical properties of C terminus proteins |
|-------------------------------------------|
| **Protein**    | $R_s$ | $\mathrm{sp}_{a,w}$ | Calculated $M_r$ | Polypeptide $M_r$ | Association state | Frictional coefficient ($f/fo$) |
|----------------|-------|---------------------|-----------------|-------------------|------------------|--------------------------|
| MC1            | 6.0   | 4.9                 | 119,188         | 57,398            | Dimer            | 1.85                     |
| GST/MC1        | 8.4   | 6.05                | 156,971         | 83,388            | Dimer            | 1.77                     |
| MC5            | 4.3   | 4.65                | 81,060          | 47,660            | Dimer            | 1.45                     |
| GST/MC5        | 5.8’  | 8.2                 | 192,808         | 73,660            | Mix of dimers &| 1.29                     |
|                |       |                     |                 |                   | tetramers        |                          |
| MC6            | 2.95  | 3.7                 | 44,249          | 41,659            | Monomer         | 1.29                     |
| GST/MC6        | 4.9   | 6.05                | 120,181         | 67,659            | Dimer            | 1.45                     |

* $R_s$ is the Stokes radius in nanometers determined by FPLC gel filtration on a Superose 12 column.

* $\mathrm{sp}_{a,w}$ is the sedimentation coefficient determined from rate zonal centrifugation.

* The molecular weight ($M$) was calculated as described by Siegel and Monty (1966) using a partial specific volume ($\upsilon_p$) of 0.72 cm$^3$/g: $M = (6\pi N R_s \mathrm{sp}_{a,w} \rho)/(1 - \upsilon_p)$.

* The monomer polypeptide molecular weight was determined from the protein sequence. Additional residues at the N terminus of the proteins in expression constructs for MC1 and MC6 were taken into account. 26 kDa was added to the molecular weight for GST fusion proteins (Smith and Johnson, 1988).

* The frictional coefficient ($f/fo$) was calculated according to the following equation: $f/fo = R_s (4\pi N M_r \rho)/3$. It represents the ratio of the frictional coefficient of the molecule to the frictional coefficient of a sphere with the same volume.

* Two protein peaks with $R_s$ of 6.5 nm and 5.2 nm were resolved by gel filtration chromatography, but only one peak was observed with rate zonal centrifugation. The value used in these calculations was the average of the two $R_s$ values.
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**Fig. 3.** Electron micrographs of rotary-shadowed MC1 and GST/MC1. Protein for rotary shadowing was prepared by centrifugation on 15–40% linear glycerol gradients. Additional glycerol (10 μl per 20 μl gradient volume) was added to the peak fractions to give a final glycerol concentration of ~40%. The protein was sprayed onto mica and rotary-shadowed with platinum (Fowler and Erickson, 1979). Specimens were photographed in the electron microscope at a magnification of 50,000×, and photographs were enlarged five times for measurement and presentation. All measurements were corrected for an assumed 1 nm shell of metal. Panel A, the first column shows selected molecules consisting of a globular head and a distinct tail. The second column shows a field in which four of the molecules exhibit a head and tail, and three others appear globular with no visible tail. The third column shows molecules that appear to be paired at the ends of their tails (upper two) or along the entire tail (bottom two). Panel B, the three columns show selected fields of GST/MC1, which appears almost exclusively as a dumbbell. Bar = 40 nm.

**Fig. 4.** Diagrammatic representation of ncd. ncd has three distinct domains: an N-terminal tail is followed by a central stalk, which is joined to the globular C-terminal motor domain. The dimensions of the stalk and “head,” or motor, domains were obtained from rotary-shadowed images of the MC1 protein. The proposed functions of each of the domains are listed. The structure of the tails is based on the predicted amino acid sequence of the ncd protein.

**TABLE V**

| Protein | Motility | MT binding in solution | -Fold stimulation of MgATPase by MT |
|---------|----------|------------------------|------------------------------------|
| ncd     | Minus-end| +                      | ND                                 |
| N1      |          | +                      |                                    |
| N2      |          | +                      |                                    |
| MC1     | Diffusional | +                   | 8                                  |
| MC5     |          | +                      | 3.5                                |
| MC6     |          | +                      | 40                                 |
| GST/MC1 | Minus-end| +                      | 2                                  |
| GST/MC5 | Minus-end| +                      | 5                                  |
| GST/MC6 | (+)      | +                      | 95                                 |

MT = microtubules; ND = not determined.

**Motility data for GST/MC1 were obtained using protein prepared by ammonium sulfate precipitation from a bacterial cell lysate. The microtubule binding and ATPase activity were determined using S-Sepharose-purified protein.**

The determinants of directionality and rotation of ncd map to the C-terminal 407 residues, which contain the domain that is conserved among the kinesin family of proteins. The smallest C-terminus protein, GST/MC6, supported only very slow microtubule gliding. The slow velocity of microtubule gliding can be attributed to a stronger binding of the motor to microtubules, based on the behavior of the truncated protein in microtubule binding and Mg-ATPase assays in solution, or, alternatively, to denaturation of the motor by interaction with the glass surface. The microtubule translocation observed with GST/MC6 delimits the ncd motor domain to the C-terminal 368 amino acids of the protein.

Results of the microtubule binding/pelleting assays suggest the presence of two, and possibly three, microtubule binding regions in ncd. The N-terminal 204 residues represent an ATP-insensitive microtubule binding domain. The C terminus contains an ATP-sensitive microtubule binding region and may also contain an ATP-insensitive microtubule binding region, as indicated by the inability of added ATP to release protein bound to microtubules in the absence of ATP or presence of AMP-PNP. Further experiments will be required to rule out nonspecific charge effects and demonstrate the existence of these microtubule binding regions.
The truncated C terminus proteins (MC1, MC5, MC6) can hydrolyze MgATP in solution but do not support microtubule gliding in motility assays; thus, the ability to hydrolyze MgATP is not correlated with in vitro motility. This is probably a reflection of the more stringent requirements of the in vitro gliding assay for activity. The GST/MC6 protein shows a microtubule-stimulated MgATPase activity that is >10 times higher than that of GST/MC1 or GST/MC5, which translocates with velocities similar to that of near full-length ncd, but GST/MC6 exhibits no, or very slow, movement on microtubules. The basal levels of MC6 and GST/MC6 MgATPase activity, however, do not differ significantly from those of the other C terminus proteins. The effect of truncation of the protein on MgATPase activity has also been observed for kinesin: a 45-kDa proteolytic fragment showed 4-5-fold increased microtubule-stimulated MgATPase activity compared with the intact protein (Kuznetsov et al., 1989).

The very high microtubule-stimulated MgATPase activities observed for MC6 and GST/MC6 suggest that a domain required for regulating ATPase activity is missing in these proteins. These two proteins lack most of the central stalk region of ncd but retain the C-terminal 308-residue mechanochanical domain. Hackney et al. (1992) propose that interaction of the kinesin tail with the motor domain regulates the ATPase activity. In ncd, a portion of the stalk, either by physically blocking the ATPase site or by dimerization of the motor domain, may regulate the ability of nucleotide to diffuse in and out of the binding/hydrolysis site when the motor is bound to microtubules. Dimerization of the motor domain might reduce the ATPase activity due to the conformation of the two heads when they are brought together by the coiled-coil stalk region. Alternatively, the GST/MC6 and MC6 proteins may be unable to release microtubules during the ATP hydrolysis cycle, and the motor in the “microtubule-stimulated” state may be capable of hydrolyzing multiple ATP molecules before release occurs. This would represent a state in which the motor power stroke is uncoupled from the ATP hydrolysis, or force, cycle. Quantitative measurement of the kinetic steps in the hydrolysis cycle will be required to determine the basic of the MgATPase activity of the truncated proteins.

MC6 and GST/MC6 showed greater binding to microtubules in the presence of MgATP in microtubule pelleting assays. These results support the idea that a longer time of binding, or stronger binding, to microtubules is directly correlated with the microtubule-stimulated MgATPase activity and inversely correlated with the ability to support microtubule gliding in vitro assays. The central stalk region of the ncd protein may function to prevent tight binding by the motor to the microtubule, allowing translocation of the motor with each ATP hydrolysis. This proposed function of the central stalk of ncd, if correct, is likely to be important in understanding motor function. A region of heptad leucine repeats from residue 199-247 was identified in the predicted sequence of ncd (Endow et al., 1990). Re-examination of the sequence reveals that the region of heptad repeats of hydrophobic residues, with leucine frequently present in position “d,” extends from amino acids 199-355 with a few interruptions. The heptad repeats are characteristic of α-helical coiled-coils and can mediate dimerization of proteins (Hodges, 1992). The MC1 protein may contain as many as 19 repeats, and MC5 may contain ~7 repeats. Both proteins exist as dimers in solution (Table IV). MC6 contains only 23 residues that may comprise part of the heptad repeat region. These residues are apparently not sufficient to support the formation of a coiled-coil (Hodges, 1992), since hydrodynamic properties of the protein indicate that MC6 exists in solution as a monomer. Our finding that both MC1 and MC5 are dimers indicates that the heptad repeat region in ncd is involved in protein dimerization and that the third protein probably exists as a dimer in vivo. Fig. 4 shows a model of the ncd protein based on our structural and functional analysis, in which ncd, like kinesin, has an elongated central stalk joined to a globular motor domain.

Models for kinesin motor function are based on the observation that native kinesin is a heavy chain dimer. Such models propose “hand-over-hand” (Schnapp et al., 1990) or “stroke-release” mechanisms for kinesin motor function (Block et al., 1990). The finding that the dimeric GST/MC1 protein is capable of motor function with properties that resemble ncd in velocity, directionality, and ability to generate torque, supports models that are based on alternating or coordinated function of a “two-headed” molecule.

Characterization of the microtubule binding properties of the N terminus of ncd and the effect of deletion of the central region on microtubule-stimulated ATPase activity provide information regarding the in vivo function of the ncd motor. The mapping of motor functions, including ATPase activity, microtubule binding, and microtubule translocation to the C-terminal mechanochanical domain extend our understanding of the in vivo function of ncd and establish a basis for studies of the mechanochanical function of the ncd motor protein. Further studies will include genetic and cell biological analysis of truncated ncd proteins, in order to confirm the results of in vitro assays.

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REFERENCES

Block, S. M., Goldstein, L. S. B., and Schnapp, B. J. (1990) Nature 348, 348-352
Brown, N. H., and Kafatos, F. C. (1988) J. Mol. Biol. 203, 425-437
Chandra, R., and Endow, S. A. (1993) Methods in Cell Biology: Microscopy: Methods in Cell Biology Motor Proteins (Schley, J., ed) Vol. 69, in press
Chandra, R., Endow, S. A., and Salmon, E. D. (1993) J. Cell Sci. 104, in press
Cohn, S. A., Ingold, A. L., and Scholey, J. M. (1987) Nature 328, 160-163
Davis, D. G. (1990) Genetics 125, 594-597
de Cuvelas, M., Tao, T., and Goldstein, L. S. B. (1992) J. Cell Biol. 116, 957-965
Endow, S. A. (1991) Trend Biochem. Sci. 16, 221-225
Endow, S. A., and Titus, M. A. (1992) Annu. Rev. Cell Biol. 8, 29-66
Endow, S. A., Henikoff, S., and Soler-Niedziesia, L. (1990) Nature 345, 81-83
Fowler, W. E., and Enckos, H. P. (1979) J. Mol. Biol. 134, 241-248
Hackney, D. D., Levitt, D. J., and Suhlan, J. (1992) J. Biol. Chem. 267, 8696-8701
Hataumi, M., and Endow, S. A. (1992a) J. Cell Sci. 104, 547-559
Hataumi, M., and Endow, S. A. (1992b) J. Cell Sci. 103, 1013-1026
Higashijima, T., Ferguson, K. M., Smigel, M. D., and Gilman, A. G. (1987) J. Biol. Chem. 262, 757-761
Hirokawa, N., Pfister, K. K., Yurifuli, H., Wagner, M. C., Brady, S. T., and Bloom, G. S. (1989) Cell 68, 861-878
Hisanaga, S., Murofushi, H., Okubara, K., Sato, R., Masuda, Y., Seki, S., and Hirokawa, N. (1989) Cell Motil. Cytoskeleton 12, 264-272
Hodges, R. S. (1992) Current Biol. 2, 122-134
Hymen, A., Dreichel, D., Kellogg, D., Sailer, S., Sawin, K. E., Steffen, P., Wordeman, L., and Mitchison, T. (1991) Methods Enzymol. 196, 478-485
Kimble, M., and Church, K. (1985) J. Cell Sci. 62, 301-318
Kuznetsova, S. A., Vaisberg, Y. A., Rothwell, S. W., Murphy, D. B., and Gelfand, N. I. (1989) J. Biol. Chem. 264, 5808-5815
Lemmon, U. K. (1970) Nature 227, 680-685
Landshultz, W. H., Johnston, P. F., and McKnight, S. L. (1988) Science 240, 1759-1764
Lewis, E. B., and Gencarella, W. (1952) Genetics 37, 600-601
Martin, B. G., and Apes, B. N. (1961) J. Biol. Chem. 236, 1372-1379
McDonald, H. B., and Goldstein, L. S. B. (1990) Cell 61, 991-1000
McDonald, H. B., Stewart, R. J., and Goldstein, L. S. B. (1990) Cell 63, 1159-1163
Meluh, P. B., and Rose, M. D. (1990) Cell 60, 1029-1041
Nayvorse, F., Niclas, J., Booher, N. H., Sparks, L., Bernstein, H. D., McCaffrey, G., and Vale, R. D. (1992) J. Cell Biol. 117, 1265-1273
Nislow, C., Lombillo, V. A., Kuriyama, R., and McIntosh, J. R. (1992) Nature 359, 543-547
Domains of the ncd Motor Protein

O'Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) Science 254, 539-544
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
Sawin, K. E., LeGuellec, K., Philippe, M., and Mitchison, T. J. (1992) Nature 359, 540-543
Schnapp, B. J., Crise, B., Sheetz, M. P., Reese, T. S., and Khan, S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 10053-10057
Siegel, L. J., and Monty, K. J. (1986) Biochim. Biophys. Acta 87, 346-362
Smith, D. R., and Johnson, K. S. (1988) Gene (Amst.) 67, 31-40
Sturtevant, A. H. (1929) Z. Wiss. Zool. 135, 323-356
Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. P., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988) J. Cell Biol. 107, 1437-1446
Walker, R. A., Salmon, E. D., and Endow, S. A. (1990) Nature 347, 780-782
Way, M., Pope, P., Gooch, J., Hawkins, M., and Weeds, A. G. (1990) EMBO J. 9, 4103-4109
Yang, J. T., Laymon, R. A., and Goldstein, L. S. B. (1989) Cell 56, 879-888

Turner, R., and Tjian, R. (1989) Science 243, 1689-1694
Vale, R. D., and Toyoshima, Y. Y. (1988) Cell 52, 459-469
Wagner, M. C., Pflaster, K. K., Bloom, G. S., and Brady, S. T. (1989) Cell Motil. Cytoskeleton 12, 185-215
Wald, H. (1936) Genetics 21, 264-279
Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. P., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988) J. Cell Biol. 107, 1437-1446
Walker, R. A., Salmon, E. D., and Endow, S. A. (1990) Nature 347, 780-782
Way, M., Pope, P., Gooch, J., Hawkins, M., and Weeds, A. G. (1990) EMBO J. 9, 4103-4109
Yang, J. T., Laymon, R. A., and Goldstein, L. S. B. (1989) Cell 56, 879-888