Binding Sites on Microtubules of Kinesin Motors of the Same or Opposite Polarity†

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ABSTRACT: The kinesin motor proteins translocate toward either the plus or minus end of microtubules (MTs). Competitive microtubule binding assays were carried out with monomeric motor domains of the minus-end-directed nonclaret disjunctional (Ncd) and Kar3 and the plus-end-directed kinesin heavy chain (KHC) to determine whether motors of the same or opposite polarity compete for binding sites on MTs and to test the idea that motor polarity is determined by differences in binding sites on MTs of the motors. The stoichiometries of binding were approximately 1 motor:1 tubulin heterodimer for all three motors. Ncd and Kar3, both minus-end motors, severely inhibited the binding of one another to MTs, as predicted theoretically for binding of the two motors to the same site on MTs, indicating that the binding sites on MTs of Ncd and Kar3 are the same or overlap extensively. Motors of opposite polarity, KHC and Ncd or KHC and Kar3, showed partial or complete inhibition of binding to MTs under different experimental protocols. The differences in binding behavior could be due to experimental conditions or be inherent in the nature of motor binding to MTs. Alternatively, differences in KHC and Ncd or Kar3 binding sites on MTs may exist such that the motors bind to partially overlapping but nonidentical sites on MTs. These differences in binding sites may be related to the opposite polarity of translocation on MTs of the motors.

MT[1] motor proteins of the kinesin family show ATP-dependent mechanochemical motility in vitro (Vale et al., 1985). Many intracellular movements, such as vesicle/organelle transport, spindle assembly, and chromosome movement, rely on kinesin motor force generation or unidirectional movement of kinesin motors on microtubules (MTs) (Bloom & Endow, 1995). Unlike other families of molecular motor proteins, kinesin family members exhibit both polarities of movement on their polymer substrate, moving either toward MT plus or minus ends. Although the basis of motor polarity is not yet understood, there exists a correlation between motor polarity and overall structure of the kinesin motor protein. Kinesins such as *Drosophila* kinesin heavy chain (KHC) (Yang et al., 1989) and *Xenopus* Eg5 (Sawin et al., 1992) have motor domains at the N-terminus of the polypeptide chain and move toward MT plus ends, while *Drosophila* Ncd (Walker et al., 1990) and *Saccharomyces* Kar3 (Endow et al., 1994) have C-terminal motor domains and move toward MT minus ends.

But the relationship between the location of the motor domain in the protein and motor polarity may not be a strict one. Using fusion proteins with various truncations of *Drosophila* KHC and Ncd, Stewart et al. (1993) and Chandra et al. (1993) found that motility and directionality are intrinsic to the motor domain. Furthermore, chimeric proteins in which the KHC motor domain was located at the C-terminus either of glutathione *S*-transferase (GST) or α-spectrin translocated toward the plus ends of MTs, like native kinesin, but with much slower velocity (Stewart et al., 1993), implying that the location of the motor within the protein primary sequence is not a determinant of motor polarity.

On the basis of the similarity in kinetic properties of KHC and Ncd (i.e., that ADP release from the motor−MT complex appears to be the rate-limiting step in the ATPase cycle for both motors), a structural rather than a biochemical model for polarity reversal of the KHC and Ncd motors was proposed (Lockhart & Cross, 1994). In this model, the two motors differ only in their initial, weak binding to MTs, which causes Ncd to move in one direction and KHC to move in the other. The nature of such a structural bias that might serve as a determinant of motor polarity is not clear. One possibility is that kinesins of opposite polarity bind to different sites on MTs, while motors of the same polarity bind to the same site. To test this hypothesis, we assayed the ability of the Ncd, Kar3, and KHC motor domains to compete for binding sites on MTs in vitro.

EXPERIMENTAL PROCEDURES

Motor Domain Proteins. The Ncd motor domain construct (MC6) has been reported previously (Chandra et al., 1993). The Kar3 and KHC motor domain sequences were amplified using the polymerase chain reaction (PCR) from GST/Kar3 (Endow et al., 1994) or a *Drosophila* cDNA library (Brown & Kafatos, 1988), respectively, and cloned into pMW174 (Way et al., 1990). The KHC motor domain protein corresponds to residues 1−337 of *Drosophila* KHC, the Kar3 motor domain corresponds to residues 383−729 of *S. cerevisiae* Kar3, and the Kar3+N11 motor domain corresponds to residues 372−729 of Kar3. DNA sequence analysis of the pMW/Kar3, pMW/Kar3+N11, and pMW/KHC constructs was carried out to eliminate the possibility of PCR missense mutations. The plasmids were found to encode wild-type motor domain proteins of 347 amino acid residues for Kar3, 358 residues for Kar3+N11, and 337 residues for KHC, with changes of $L_{383} \rightarrow M$ in Kar3 and

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[1] Abbreviations: MT, microtubule; KHC, kinesin heavy chain; Ncd, nonclaret disjunctional; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; GST, glutathione *S*-transferase.

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$V_{372} \rightarrow$ M in Kar3+N11 for translation initiation and $S_2 \rightarrow$ G and $A_3 \rightarrow$ S in KHC during plasmid construction.

Expression and Purification of Motor Proteins. Plasmids were transformed into competent BL21(DE3)pLysS host cells (Studier et al., 1990) for the expression of protein. pMW/Kar3 and pMW/Kar3+Nll were cotransformed with pACYC184/*argU*$^+$ (Spanjaard et al., 1990). *argU*$^+$ encodes *Escherichia coli* tRNA Arg_{AGA}, which is limiting in *E. coli* and has been shown to increase recombinant gene expression when supplemented by cotransfection (Brinkman et al., 1989). The Kar3 motor domain contains 10 Arg_{AGA} codons. Kar3 protein, purified after growth with *argU*$^+$, showed greater homogeneity than protein purified after growth without *argU*$^+$ upon analysis by electrospray ionization mass spectroscopy (R. Stevens, H. Song, S. A. Endow, & T. Oas, unpublished observations), but there was no evidence for amino acid substitutions in protein purified after growth with or without *argU*$^+$. Expression of motor proteins was induced by addition of 0.4 mM IPTG followed by growth at 18 °C for 6 h. Proteins were purified by chromatography on S-Sepharose, as described (Chandra et al., 1993). Purification to homogeneity was accomplished by FPLC on Mono S (Kar3 and Kar3+N11) and/or Superose 12 (Ncd, Kar3, Kar3+N11, and KHC).

Purification of Tubulin and Preparations of MTs. Tubulin was purified from porcine brains, as described previously (Williams & Lee, 1982). MTs were assembled by adding GTP and stabilized with taxol (40 μM final concentration). MTs were depleted of GTP by centrifugation at 100000*g* for 30 min at 37 °C followed by resuspension in HEM buffer (10 mM Hepes, pH 7.2, 1 mM EGTA, 1 mM $MgCl_2$) and 40 μM taxol.

MT Binding Assays. Motor proteins were clarified by centrifugation at 100000*g* for 30 min at 2 °C prior to the MT binding assays. Various concentrations (1, 2, 3, 5, 7, 9, and 11 μM) of each motor domain were incubated in HEM plus 50 mM NaCl, 10 μg of monomeric bovine serum albumin (Pentex, Miles Laboratory, Inc.), 40 μM taxol, and 3 or 4 μM GTP-depleted and taxol-stabilized MTs in 100 μL final volume. The mixtures were gently vortexed and incubated for 3 min at room temperature. The MT-bound and unbound motor were separated by centrifugation at 100000*g* for 30 min at 22 °C in a Beckman TLX ultracentrifuge. Supernatants were removed, the pellets were resuspended in 100 μL of 1× electrophoresis sample buffer, and the supernatant and the pellet were analyzed by SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue. Bands on wet gels were quantitated using a scanning laser densitometer (Ultroscan, LKB). The OD of scanned bands was found to increase linearly with the amount of protein loaded on the gel for 0.1−2 μg of protein. This method of quantitation was comparable to, but more sensitive than, analysis of digital images. The MT-bound motor was plotted against the unbound motor, and the data were fit to a rectangular hyperbola using Kaleidagraph v.3.0. In control incubations of combined motor proteins without MTs, equivalent or less protein pelleted after centrifugation than in incubations of the single motors without MTs, indicating that there was no significant interaction between the two motor domain proteins during the incubation that caused nonspecific precipitation or aggregation. This control experiment of the combined motors without MTs was carried out for all of the competitive assays.

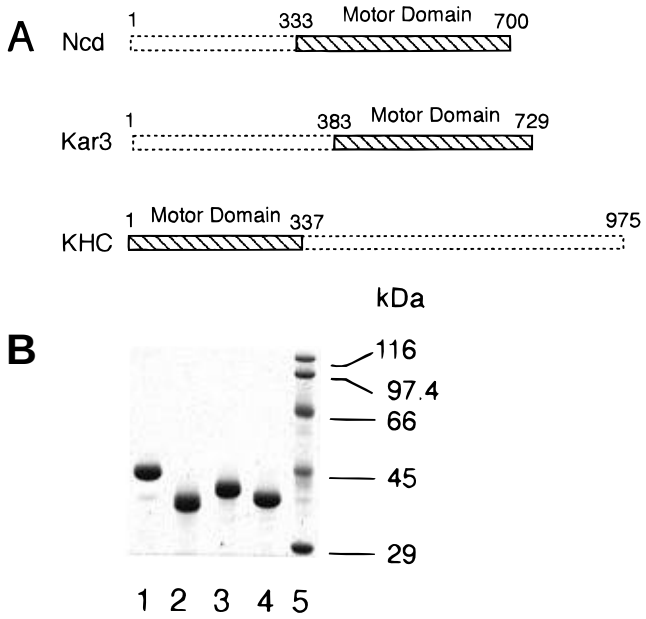

FIGURE 1: Structures of MT motors and SDS-PAGE of purified motor domain proteins. (A) Motor domains (hatched boxes) contain conserved ATP- and MT-binding regions. The numbers denote amino acids starting from the N-terminus. Kar3+N11 (not shown) extends from amino acid 372−729 of Kar3. (B) SDS-PAGE of purified motor domain proteins. Two micrograms of each protein was electrophoresed on a 10% gel and visualized by staining with Coomassie Blue. Lane 1, *Drosophila* Ncd motor domain; lane 2, *Saccharomyces* Kar3 motor domain; lane 3, Kar3+N11; lane 4, *Drosophila* kinesin heavy chain motor domain; lane 5, molecular mass markers in kilodaltons.

Protein Concentration. Protein concentration determinations were critical for these experiments. Protein concentrations (except KHC) were determined using molar extinction coefficients calculated from the protein amino acid composition, assuming that 1 molecule of ADP or guanosine nucleotide is bound to each molecule of motor protein or tubulin monomer, and OD_{280} values of the motor domain proteins or tubulin in 6 M guanidine hydrochloride (Gill & von Hippel, 1989). Protein concentrations were routinely determined using the Bio-Rad protein assay reagent and corrected using the factor determined from the calculated extinction coefficient and absorbance in 6 M guanidine hydrochloride. The absorbance of KHC in 6 M guanidine hydrochloride showed wide variability; the concentration of KHC was therefore determined using the Bio-Rad reagent alone.

RESULTS

Purification and Characterization of Motor Domain Proteins. To minimize steric hindrance (Kikkawa et al., 1994), minimal motor domains necessary for MT binding and ATP hydrolysis were constructed (Figure 1A), including two forms of Kar3, Kar3 and Kar3+N11. Motor domain proteins were expressed in bacteria and purified to near homogeneity (>95%) (Figure 1B). Biochemical and physical properties of the motor proteins are summarized in Table 1. The retention times on a Superose 12 FPLC column for highly purified Kar3, Kar3+N11, and KHC motor proteins were longer than that of monomeric bovine serum albumin (Pentex, Miles Laboratory, Inc.) in the presence of 200 mM NaCl and were the same as that of the Ncd motor domain, previously determined to be monomeric (Chandra et al.,

Table 1: Physical and Biochemical Properties of Motor Domain Proteins[a]

motor	polarity	M_r[b] (Da)	association state[c]	k_{cat}[d] (s^{-1})	K_d[e] (μM)
Ncd	minus	41 600	monomer	4.3	2.32 ± 0.39
Kar3	minus	38 910	monomer	0.65	1.36 ± 0.13
KHC	plus	37 360	monomer	30	0.46 ± 0.15

[a] The proteins used in these experiments were bacterially-expressed motor domains. [b] Molecular mass was calculated from the amino acid sequences predicted from the nucleic acid sequences. [c] Association states of Ncd and KHC have been reported previously (Chandra et al., 1993; Huang et al., 1994). Kar3 was determined to be a monomer by its retention on a Superose 12 column. [d] k_{cat} was determined using ATPase assays (Huang & Hackney, 1994). [e] Dissociation constant from MTs (K_d) was determined from MT pelleting assays ($n = 4-6$) (Huang & Hackney, 1994) without added nucleotide and with modifications.

1993), indicating that all of the purified motor domain proteins are monomeric. A KHC motor domain protein three amino acids longer than ours, DKH340, was previously reported to be a monomer (Huang et al., 1994), consistent with this conclusion. The Kar3+N11 ATPase specific activity and dissociation constant (K_d) from MTs are essentially the same as those of the Kar3 motor domain, but its greater M_r permits Kar3+N11 to be resolved from the Kar3 and KHC motor domain proteins by gel electrophoresis (Figure 1B).

MT Binding Assays. MT binding assays with single-motor proteins were carried out to determine K_d values and binding stoichiometries of the motors to MTs. The binding profiles were obtained by plotting MT-bound vs unbound motor. Control experiments without MTs were performed to correct for the motor that pelleted under binding assay conditions due to aggregation (hence unbound). This was found to be a significant problem only for the Ncd motor domain (~10−25% of the protein in the reaction mixture pelleted in the absence of MTs). Higher concentrations of salt and incubations at a lower temperature could alleviate the aggregation problem (Moore et al., 1996), but these conditions were not suitable for the competitive MT binding assays due to the greatly reduced binding of the motors to MTs. For data analysis, the amount of motor that pelleted in the absence of MTs was subtracted from the pellet in the presence of MTs and added to the unbound motor fraction. Subtraction of the motor that pelleted in the absence of MTs from the total amount in the reaction rather than adding it to the unbound fraction resulted in K_d values that differed significantly from the values that we report in Table 1 but did not have a significant effect on the shape of the observed binding profiles. The theoretical binding profiles were somewhat shifted, but this method of analysis did not alter the conclusions of any of the combined motor experiments. Under the assay conditions, the binding stoichiometries of the motors to MTs were ~1 motor:1 tubulin dimer for all three motors, including Kar3+N11.

Positive Control for Competitive Binding Assays. The competitive MT binding assays were designed to determine the effect of a given motor domain protein on the binding to MTs of a second motor domain protein. First, as a positive control, competitive assays were carried out between Kar3 and Kar3+N11, which bind to the same site on MTs. The bound and unbound Kar3 (pellet and supernatant, respectively) were visualized by staining gels with Coomassie Blue

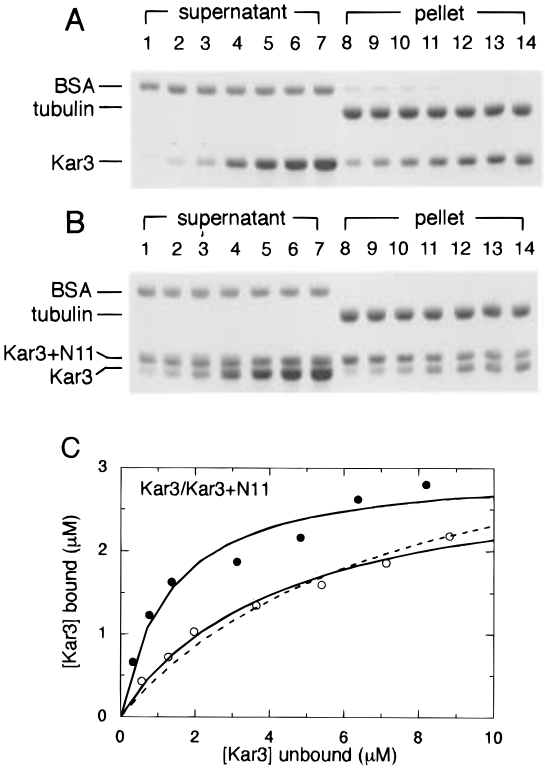

FIGURE 2: Competitive MT binding between Kar3 and Kar3+N11. MT binding assay conditions were as described in Experimental Procedures. (A) SDS-PAGE of unbound and MT-bound Kar3. Kar3 motor protein (1, 2, 3, 5, 7, 9, and 11 μM) was coincubated with 3 μM MTs [tubulin heterodimer], and unbound (lanes 1−7, 10 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (B) SDS-PAGE of unbound and MT-bound Kar3 in the presence of Kar3+N11. The same conditions as A, but 4 μM Kar3+N11 was added to each concentration of Kar3. (C) MT binding profiles of Kar3 in the presence and absence of Kar3+N11. The binding profiles of Kar3 without Kar3+N11 (●), Kar3 with Kar3+N11 (○), and theoretical competitive binding (- - -) are shown. The theoretical binding profile was calculated by the formula (Hammes, 1982)

$$r_1 = \frac{nL_1/K_1}{(1 + L_1/K_1 + L_2/K_2)}$$

where r_1 = moles of Kar3 bound per mole of MTs in the presence of Kar3+N11 ([MT] = 3 or 4 μM in the experiments described here); $n = 1$, the number of Kar3 binding sites per tubulin dimer; L_1 = unbound Kar3 in the absence of Kar3+N11; L_2 = unbound Kar3+N11 in the absence of Kar3; $K_1 = 1.36$ μM, the dissociation constant of Kar3 from MTs; and $K_2 = 1.41$ μM, the dissociation constant of Kar3+N11 from MTs. Dissociation constants were determined by single-motor MT binding assays ($n = 4-6$).

(Figure 2A). The MT binding profile of Kar3 (1−11 μM) in the presence of 4 μM Kar3+N11 (Figure 2B) was nearly identical to the theoretical competitive curve for binding of the two motors to the same site on MTs (Figure 2C) and showed a large deviation from the binding profile for Kar3 (1−11 μM) alone. When lower concentrations of Kar3+N11, either 1 or 2 μM, were incubated with 1−11 μM Kar3 in competitive assays, the binding profiles of Kar3 to MTs were again nearly identical to the theoretical competitive profiles for binding of the motors of the same site on MTs. The Kar3 and Kar3+N11 motor proteins are expected to compete with one another for binding sites on MTs; these results therefore provide confidence in our methods.

Competitive Assays between Kar3 and Ncd. Competitive assays of the same design were carried out between Kar3

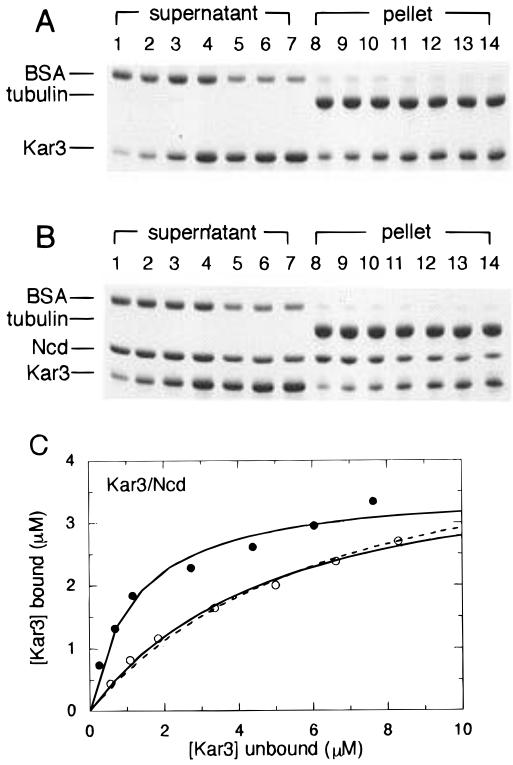

FIGURE 3: Competitive MT binding between the Kar3 and Ncd motor domain proteins. The same experimental design as in Figure 2, but with Ncd instead of Kar3+N11. (A) SDS-PAGE of unbound and MT-bound Kar3. Kar3 motor protein (1, 2, 3, 5, 7, 9, and 11 μM) was coincubated with 3 μM MTs [tubulin heterodimer], and unbound (lanes 1−4, 10 μL; lanes 5−7, 5 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (B) SDS-PAGE of unbound and MT-bound Kar3 in the presence of Ncd. Ncd (4 μM) was added to each concentration of Kar3 as described in A, and unbound (lanes 1−4, 10 μL; lanes 5−7, 5 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (C) MT binding profiles of Kar3 in the presence and absence of Ncd. The binding profiles of Kar3 without Ncd (●), Kar3 with Ncd (○), and theoretical competitive binding (- - -) are shown. The theoretical binding curve was obtained by the formula described in Figure 2C, where $n = 1$, $K_{1,\mathrm{Kar3}} = 1.36$ μM, and $K_{2,\mathrm{Ncd}} = 2.32$ μM and L_1 and L_2 were obtained from the experimental data.

and Ncd motor domains from kinesin proteins with the same polarity of translocation on MTs. Variable amounts of Kar3 (1−11 μM) were incubated with or without 4 μM Ncd, and MT-bound and unbound fractions were separated by centrifugation. The free motor concentrations of 3 μM Kar3 and 4 μM Ncd in single-motor binding assays were 1.3 μM ($n = 3$) and 2.2 μM ($n = 4$), respectively. These free motor concentrations approximate the K_d values of the motors, providing conditions with the greatest sensitivity to competition between the motors. The MT-bound and unbound motor fractions again correspond to pellets and supernatants, respectively, on the gel (Figures 3A and 3B). The resulting binding profiles of Kar3 to MTs with or without Ncd are shown in Figure 3C. The binding of the Kar3 motor domain in the presence of the Ncd motor domain was severely inhibited (Figure 3C). Moreover, the binding profile of Kar3 in the presence of Ncd was nearly identical to the theoretical competitive profile for binding of the Kar3 and Ncd motor domains to the same site on MTs (Figure 3C). In addition, in reversed competition assays, the MT binding profile of Ncd (1−11 μM) in the presence of 3 μM Kar3 was essentially identical to the theoretical competitive binding curve (data not shown). These results demonstrate that the binding sites on MTs of Kar3 and Ncd are the same, or overlap extensively.

Competitive Assays between KHC and Kar3. Since the migration behavior of the Kar3 motor domain protein on SDS-PAGE was very similar to that of KHC (Figure 1B), Kar3+N11 was used in place of Kar3 in competition assays with KHC. In these assays, the effect of each of the proteins on the binding to MTs of the other was tested. Variable amounts of KHC (1−11 μM) were incubated with 3 μM Kar3+N11, or variable amounts of Kar3+N11 (1−11 μM) were incubated with 2 μM KHC, and MT-bound and unbound fractions were separated by centrifugation. The unbound motor concentrations of 2 μM KHC and 3 μM Kar3+N11 in single-motor binding assays were 0.3 μM ($n = 3$) and 1.1 μM ($n = 3$), respectively, close to the K_d values of the motors. The MT-bound and unbound motor fractions again correspond to pellets and supernatants, respectively, on the gel (Figure 4). The binding of the KHC motor domain to MTs was partially inhibited by the Kar3+N11 motor domain (Figure 4C), but the competitive MT binding curve for KHC in the presence of Kar3+N11 was not identical to the theoretical competitive binding profile (Figure 4C). This result was reproducible using 4 μM Kar3+N11. In these experiments, the displacement of Kar3+N11 from MTs increased as the amount of bound KHC increased (Figure 4B). These results indicate that the binding sites of the two motors are probably overlapping, but not identical. In contrast, the binding profile of Kar3+N11 to MTs in the presence of KHC was almost identical to the theoretically predicted competitive binding profile (Figure 4F), implying that the KHC and Kar3 motor domains bind to the same sites on the MT. These two results are not in accord with one another and will be discussed in the Discussion section.

Competitive Assays between KHC and Ncd. Competitive assays of the same design as for KHC and Kar3+N11 were carried out between the KHC and Ncd motor domains. Variable amounts of KHC (1−11 μM) were incubated with 4 μM Ncd, or variable amounts of Ncd (1−11 μM) were incubated with 2 μM KHC, and MT-bound and unbound fractions were separated by centrifugation. The unbound motor concentrations of 4 μM Ncd and 2 μM KHC in single-motor binding assays were 2.2 μM ($n = 4$) and 0.3 μM ($n = 3$), respectively, approximating the K_d values of the motors. The MT-bound and unbound motor fractions again correspond to pellets and supernatants, respectively, on the gel (Figure 5). The binding profiles of KHC to MTs with or without Ncd and Ncd with or without KHC are shown in Figures 5C and 5F, respectively. The binding of Ncd to MTs is inhibited by KHC, as predicted theoretically for binding of the motors to the same site on MTs. However, the binding curve for KHC in the presence of Ncd differs from the theoretical competitive binding profile, as observed in the experiment between KHC and Kar3+N11. This result was reproducible and observed in extensively repeated experiments. The order of addition of the two motor proteins had no effect on the results; the binding profiles for KHC in the presence of Ncd deviated from the theoretical competitive binding curve when either KHC or Ncd was added first to MTs.

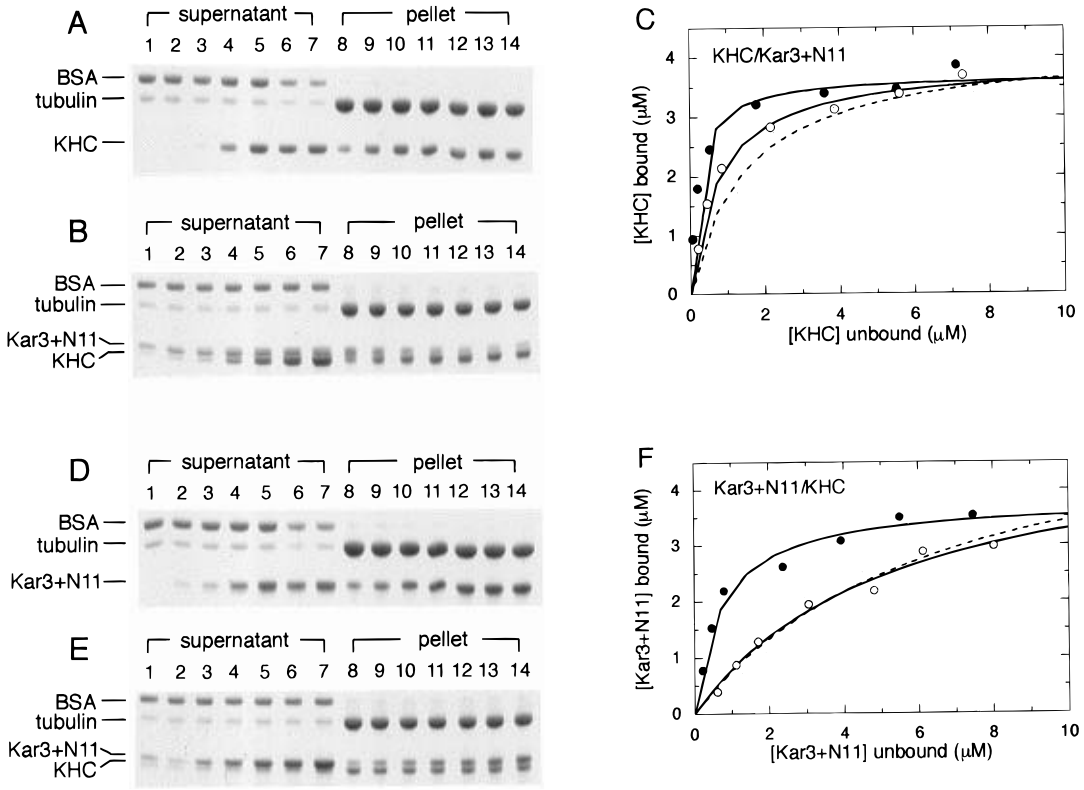

FIGURE 4: Competitive MT binding between the KHC and Kar3+N11 motor domain proteins. The same experimental design as in Figure 2, but with KHC instead of Kar3 (A−C). (A) SDS-PAGE of unbound and MT-bound KHC. KHC motor protein (1, 2, 3, 5, 7, 9, and 11 μM) was coincubated with 4 μM MTs [tubulin heterodimer], and unbound (lanes 1−5, 10 μL; lanes 6−7, 5 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (B) SDS-PAGE of unbound and MT-bound KHC in the presence of Kar3+N11. Kar3+N11 (3 μM) was added to each concentration of KHC described in A, and unbound (lanes 1−7, 5 μL) and MT-bound fractions (lanes 8−14, 5 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (C) MT binding profiles of KHC in the presence and absence of Kar3+N11. The binding profiles of KHC without Kar3+N11 (●), KHC with Kar3+N11 (○), and theoretical competitive binding curve (- - -) are shown. The theoretical binding curve was obtained by the formula described in Figure 2C, where $n = 1$, $K_{1,\mathrm{KHC}} = 0.46$ μM, and $K_{2,\mathrm{Kar3+N11}} = 1.41$ μM and L_1 and L_2 were obtained from the experimental data. (D−F) The experimental design was the same as the competition experiment described above, but KHC and Kar3+N11 were reversed. The concentration of the KHC motor domain used was 2 μM rather than 3 μM to produce a free motor concentration close to the K_d of KHC.

DISCUSSION

The problem of polarity reversal in the kinesin motor proteins has attracted much interest. Two major classes of models, biochemical and structural, have been proposed to explain the opposite polarity of translocation on MTs of different kinesin motors (Endow, 1995). Assuming that the rate-limiting step in the ATPase cycle for both Ncd and KHC is ADP release and the effects of adenosine nucleotides on the affinities of the motors to MTs are similar, Lockhart and Cross (1994) attributed the reversed polarity of Ncd relative to KHC to structural rather than biochemical differences. We report here that differences in MT binding sites may exist between plus-end-directed and minus-end-directed kinesin motors, as revealed by differences in competition assays, that could contribute to the directional reversal of the motors.

The results of our competitive MT-binding experiments provide evidence that the Kar3 and Ncd motor proteins, which show the same polarity of movement on MTs, bind to the same or extensively overlapping sites on MTs. The other combinations of motors, KHC and Kar3 or KHC and Ncd, showed severe inhibition by KHC of Kar3 or Ncd binding to MTs, but the binding profiles of KHC in the presence of either Kar3 or Ncd differed from the theoretical competitive profiles, showing less inhibition than predicted for binding of the two motors to the same site on MTs. The differences in experimental results using the same combinations of motors but with KHC variable rather than fixed in concentration may be a consequence of differences in K_d values from MTs of the motors, together with the presence of trace amounts of nucleotide that copurify with the motor domain proteins and are released upon motor binding to MTs. The competitive assays for KHC and Kar3 or KHC and Ncd were carried out using free motor concentrations that approximated the K_d from MTs of the motor of fixed concentration and extended both above and below the K_d of the motor of variable concentration. These conditions should provide the greatest sensitivity for competitive binding assays. However, even under these conditions, the much lower K_d from MTs of KHC, compared to that of Kar3 or Ncd, may have led to displacement by KHC of bound Kar3 or Ncd. The inability of increasing concentrations of Kar3 or Ncd to compete with KHC for MT binding sites may reflect the bias in K_d that favors binding by KHC. The Kar3 and Ncd motor domain proteins may also show a greater sensitivity to trace amounts of nucleotide present in the assays, compared with KHC. The observed binding behavior could thus be due to experimental conditions or be inherent in the nature of motor binding to MTs. Alternatively, the results of our competition assays can be interpreted as evidence that differences in binding sites on MTs exist for motors of different polarity.

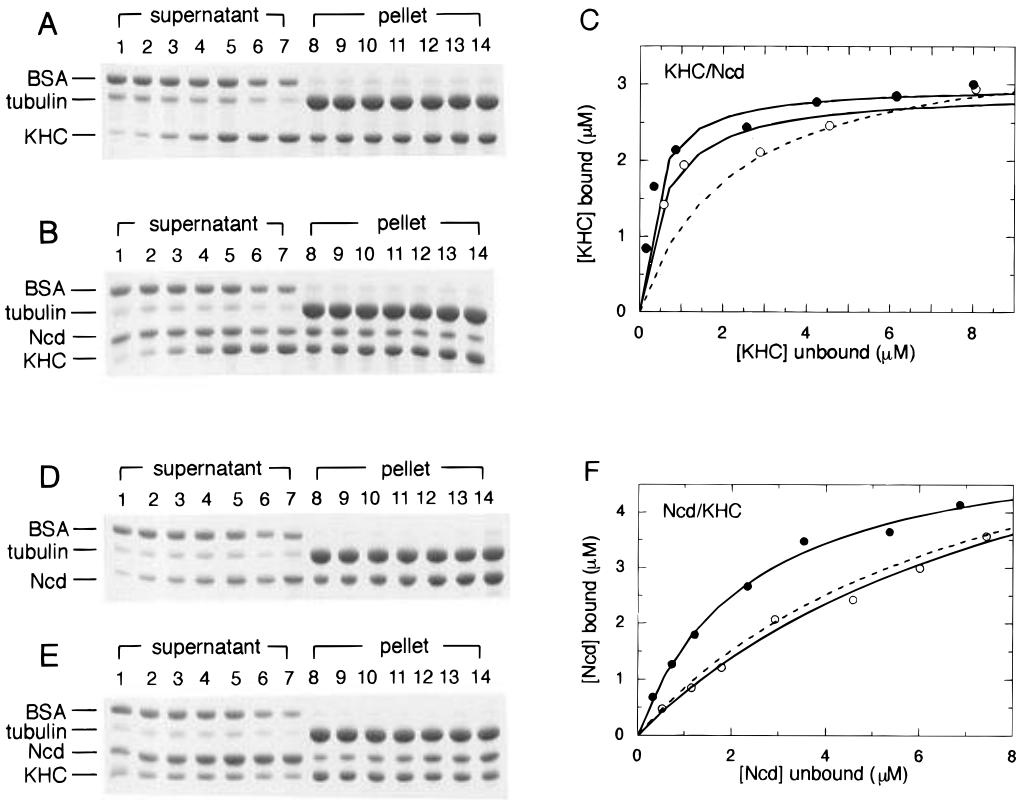

FIGURE 5: Competitive MT binding between the KHC and Ncd motor domain proteins. (A) SDS-PAGE of unbound and MT-bound KHC. KHC motor protein (1, 2, 3, 5, 7, 9, and 11 μM) was coincubated with 4 μM MTs [tubulin heterodimer], and unbound (lanes 1−5, 10 μL, lanes 6−7, 5 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (B) SDS-PAGE of unbound and MT-bound KHC in the presence of Ncd. Ncd (4 μM) was added to each concentration of KHC described in A, and unbound (lanes 1−5, 10 μL; lanes 6−7, 5 μL) and MT-bound fractions (lanes 8−14, 10 μL) were separated by SDS-PAGE and visualized by staining with Coomassie Blue. (C) MT binding profiles of KHC in the presence and absence of Ncd. The binding profiles of KHC without Ncd (●), KHC with Ncd (○), and theoretical competitive binding (- - -) are shown. The theoretical binding curve was obtained by the formula described in Figure 2C, where $n = 1$, $K_{1,KHC} = 0.46$ μM, and $K_{2,Ncd} = 2.32$ μM and L_1 and L_2 were obtained from the experimental data. (D−F) The experimental design was the same as the competition assay described above, but KHC and Ncd were reversed. The concentration of KHC motor domain used was 2 μM to produce a free motor concentration close to the K_d of KHC.

Widely differing K_d values of motors from MTs have been reported in the literature for kinesin and Ncd (Shimizu et al., 1995; Lockhart et al., 1995; Walker, 1995; Ma & Taylor, 1995). Shimizu et al. (1995) pointed out that the major forces for the interactions of motors with MTs are believed to be electrostatic and therefore the discrepancies in K_d values even from the same laboratory are probably due to different buffer conditions, including ionic strength, variations in measurements of protein concentration, and differing (trace) amounts of nucleotide in the binding reactions. Since our experiments were carried out using the same buffer conditions and the same or similar preparations of protein, the K_d values of the motors from MTs that we report are internally consistent, although they may differ from those reported by others. These differences in K_d values should not alter our basic conclusions.

Recently, Lockhart et al. (1995) reported that the binding sites for rat KHC on MTs overlap with those of *Drosophila* Ncd, a conclusion that is similar to ours. However, their conclusion was based on competitive MT binding assays using dimeric (two-headed) motor proteins of ~147 kDa for Ncd (GST-MC5) and ~90 kDa for KHC (K401), rather than monomeric motor proteins. The displacement of KHC from MTs by Ncd in their experiments can be attributed to steric effects rather than to shared binding sites of the motors on MTs. Each tubulin heterodimeric subunit of ~110 kDa is probably too small to accommodate two different motors of the size that were used, even given the possibility that the binding sites of the motors to MTs are completely different and only one of the two heads can participate in binding at a time.

Another potential problem in using dimeric motors in competitive binding assays is that the binding of two-headed motors to MTs may not be a random collision phenomenon, as is likely to be the case for monomeric motor proteins. The model of hand-over-hand movement of dimeric kinesin motors on MTs predicts that once one head of a two-headed motor is bound to MTs, the other head has a much greater chance of binding than an unbound two-headed motor in solution (Peskin & Oster, 1995; Hackney, 1995). The kinetic predictions for two-headed or dimeric motors in competition assays are therefore much more complicated than for monomeric motors.

The problem of potential steric effects that prevent binding of a second motor to a tubulin heterodimer already occupied by a bound motor probably also extends to the experiments that we report here. A tubulin heterodimer is ~8 nm in length while the dimensions of the monomeric motor domains used in our experiments are probably the same as those of the recently crystallized monomeric KHC and Ncd motor domains (Kull et al., 1996; Sablin et al., 1996), which have been estimated as 70 × 45 × 45 Å and 75 × 45 × 45 Å, respectively. The similarity in size between a tubulin heterodimer and the KHC or Ncd motor domain makes it

difficult to conceive that the two motor domains could show totally independent binding in the competitive binding assays, since the binding of the KHC and Ncd or KHC and Kar3 motor domains at a stoichiometry of 1 motor:1 tubulin heterodimer, as observed under our assay conditions, would cause displacement of motors that bind to adjacent sites on the same tubulin monomer.

Since our results were obtained using only three different motor domains, the possible contribution of different MT binding sites to directionality reversal of the motors may not be conclusive. It is, however, noteworthy that motors of the same polarity show complete competition for binding sites on MTs, while motors of opposite polarity do not show mutual exclusion of each other in competition assays, implying that the binding sites on MTs of the motors could differ but be partially overlapping or otherwise spatially inaccessible once one motor is bound. These issues should be resolvable by direct peptide mapping of binding sites on MTs of the kinesin motors.

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