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Orphan Kinesin NOD Lacks Motile Properties But Does Possess a Microtubule-stimulated ATPase Activity

Heinrich J.G. Matthies,* Ronald J. Baskin, and R. Scott Hawley†‡

Department of Genetics, Section of Molecular and Cellular Biology, University of California, Davis, California 95616

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NOD is a *Drosophila* chromosome-associated kinesin-like protein that does not fall into the chromokinesin subfamily. Although NOD lacks residues known to be critical for kinesin function, we show that microtubules activate the ATPase activity of NOD >2000-fold. Biochemical and genetic analysis of two genetically identified mutations of NOD (NOD_{DTW} and $NOD_{"DR2"}$) demonstrates that this allosteric activation is critical for the function of NOD in vivo. However, several lines of evidence indicate that this ATPase activity is not coupled to vectorial transport, including 1) NOD does not produce microtubule gliding; and 2) the substitution of a single amino acid in the *Drosophila* kinesin heavy chain with the analogous amino acid in NOD results in a drastic inhibition of motility. We suggest that the microtubule-activated ATPase activity of NOD provides transient attachments of chromosomes to microtubules rather than producing vectorial transport.

INTRODUCTION

Kinesins convert the chemical energy stored in ATP into mechanical energy for unidirectional transport along microtubules (MTs) (Brady, 1985; Block *et al.*, 1990; Romberg and Vale, 1993; Crevel *et al.*, 1996; Lockhart and Cross, 1996; Rice *et al.*, 1999; Vale and Milligan, 2000). This generation of force is based on a cycle of conformational changes, dependent upon the hydrolysis state of the bound nucleotide. For this force production step of the cycle to be effective, the kinesins must first be firmly attached to their microtubule track. This firm attachment allows force to be generated against the microtubules and is critical for unidirectional motion (Rice *et al.*, 1999). ATP is then hydrolyzed, phosphate is released, and the kinesins are left in the ADP-bound state, with a greatly reduced affinity for microtubules. This lowered affinity allows the ADP-bound kinesins to dissociate from the microtubules, thereby preventing them from producing drag, and allows other motors in the ATP-bound state to attach and produce force efficiently.

In the past few years the structural elements responsible for these cycles of conformational changes have been uncovered (Vale and Milligan, 2000). The hydrolysis state of the nucleotide appears to be monitored by two regions, which interact with the gamma phosphate of ATP, called switch I and switch II (Vale and Milligan, 2000). These gamma phosphate sensors are found in G proteins, myosins, and kinesins (Vale and Milligan, 2000). The phosphate sensor moves in response to phosphate release, and this is transmitted and amplified by the switch II helix to other parts of the motor protein. Switch II is involved in communication between the active site, the allosteric activator (polymer-binding site for kinesin and myosin), and the mechanical elements (kinesins and myosins). It is not clear which microtubule-binding regions underlay changes in microtubule affinity in various nucleotide states.

One way of learning how these structural elements function together is by studying kinesins that may use these various components to different degrees or in different manners. Because kinesins have wildly divergent biological roles, some aspects of their structure could be predicted to vary. Indeed, kinesins have been grouped into various subfamilies based upon sequence analysis, and these subfamilies typically represent kinesins with similar biological functions (Goldstein, 1993; Goodson *et al.*, 1994; Vale and Fletterick, 1997). Motor domains within a subfamily are very similar, whereas kinesins from different subfamilies are more divergent but nonetheless 35–45% identical.

Biochemical and structural studies have shown that kinesins from various subfamilies function by similar biochemical mechanisms (Hackney, 1988, Lockhart and Cross, 1994; Gilbert *et al.*, 1995; Ma and Taylor, 1995a,b; Crevel *et al.*, 1996;

* Current addresses: Department of Biology, University of Utah, 257 S. 1400 East, Salt Lake City, UT 84112-0840; †Stowers Institute for Medical Research, 1000 E. 50th St., Kansas City, MO 64110, E-mail address: RSH@Stowers-Institute.org.
‡ Corresponding author. E-mail address: RSH@Stowers-Institute.org.

Lockhart and Cross, 1996; Kull *et al.*, 1996; Sablin *et al.*, 1996; Kikkawa *et al.*, 2001). However, certain biochemical and biophysical differences, such as the efficiency of force production, have been noted among members of different subfamilies (Vale and Fletterick, 1997). The variations in these biochemical and biophysical properties allude to the diverse biological functions of the various subfamilies.

The *Drosophila* kinesin-like protein NOD falls into the orphan category and these kinesins are the most divergent members of the kinesin superfamily. Preliminary analysis of two members of this family demonstrates the unusual nature of these kinesins. The yeast protein SMY1 does not localize to microtubules in vivo, nor does it require intact microtubules to compensate for the loss of a yeast myosin (Myo2). Moreover, a mutation in the P-loop of SMY1 does not disrupt the ability of SMY1 to rescue a Myo2 mutant, nor does it mislocalize SMY1. This is true despite the observation that an analogous mutation in a more canonical kinesin strongly disrupts both function and localization (Meluh and Rose, 1990). Another member of this orphan family, Costal2 (COS2), localizes to microtubules, and binds microtubules in vitro, but this binding is ATP insensitive (Robbins *et al.*, 1997; Sissons *et al.*, 1997). These studies demonstrate that this subfamily consists of members with potentially diverse biochemical natures; however, biochemical studies of these two orphan kinesins have been difficult. To further understand the function of this orphan family, we have investigated the biochemical properties of NOD.

The NOD kinesin-like protein is comprised of two critical domains, the motor domain and the cargo-binding domain (Afshar *et al.*, 1995a,b). The first of these is a 318-amino acid region with homology to the motor domain of the kinesin superfamily (Figure 1). Genetic and molecular studies of the dominant *nod* mutation nod^{DTW} and its partial revertants have demonstrated that the motor domain of NOD is critical for its function (Rasooly *et al.*, 1991, 1994). The genetic and cytological characterizations of NOD strongly suggest that it exerts a plateward (or antipolar) force (Rasooly *et al.*, 1991, 1994; Theurkauf and Hawley, 1992; Afshar *et al.*, 1995a; Karpen *et al.*, 1996; Matthies *et al.*, 1999) and NOD is found along the surface of meiotic chromosomes (Afshar *et al.*, 1995a). Assuming that the polarity of MTs in the oocyte meiosis I spindle is canonical, NOD must act as either a plus-end–directed motor or as a brake. Curiously, Clark *et al.* (1997) came to the opposite conclusion while studying flies harboring a transgenic construct that expressed the motor domain of NOD fused to β-galactosidase via portions of kinesin (see DISCUSSION).

To bridge the gap between the existing studies of NOD and our biochemical characterization, we began by characterizing the biochemical and biophysical nature of the NOD motor domain. From these studies, we conclude that NOD functions as a brake, via its microtubule-activated ATPase activity, rather than as a motor that actually moves chromosomes along the spindle.

MATERIALS AND METHODS

Construction and Purification of Recombinant NOD Expression Constructs

The motor domain of NOD (aa 1–320) was cloned into the *Bam*HI and HINDIII sites of pGEX KG and a modified pGEX vector containing an N-terminal thrombin and an in-frame consensus cAMP-dependent (PKA) phosphorylation site (GSRRASVGS) with the use of polymerase chain reaction (PCR) and an in-frame stop codon. Use of this stop codon adds three novel C-terminal amino acids: NNS. Therefore, this construct leads to the expression of the entire NOD motor domain, including the α helix (α6), two amino acids in the adjacent region, and three random residues. Two mutants of NOD with point mutations were generated by mutagenic primers with the use of the QuikChange method (Stratagene, La Jolla, CA). The entire coding sequence was sequenced on both strands. Full-length NOD (aa 1–666) was cloned into the modified pGEX-KG vector with the use of the *Bam*HI and *Xba*I sites. A truncated version of NOD lacking the cargo-binding domain encoding residues 1–485 was subcloned into the pRSET vector by PCR and put in frame with the 87 BCCP peptide (NOD-485B), allowing in vivo biotinylation in *Escherichia coli*. This fusion with *Drosophila* kinesin heavy chain has previously been shown to allow the biotinylated kinesin to generate microtubule gliding on streptavidin-coated glass (Berliner *et al.*, 1994).

GST-NOD320, GST-NOD320$_{DTW}$, GST-NOD320$_{"DR2"}$, GST-NOD666, and NOD 485B were expressed as fusion proteins in BL21-Codon Plus cells (Stratagene). Single colonies were grown in 5 ml of overnight cultures at 20°C in LB containing both ampicillin and chloramphenicol and then diluted 1:100. Cultures were allowed to reach an OD_{600} of 0.8–1.0 and then induced with 0.3 mM isopropyl β-D-thiogalactoside. Pellets were then flash frozen and stored at −80°C. Cells were lysed in 50 mM HEPES pH 7.5, 5 mM Na_2ATP, 7 mM $MgSO_4$, 5 mM Na_2EGTA, 0.1 mM Na_2EDTA, 300 mM NaCl, 5 mm dithiothreitol (DTT) in the presence of a standard protease inhibitor cocktail with 0.1 mg/ml lyzozyme, and then incubated with 50 μg/ml DNase and RNase. The full-length 6X-his-tagged protein was purified in the absence of chelating reagents and β-mercaptoethanol was used instead of DTT. This suspension was then passed through either a French Press or microfluidizer at 11,000 psi with both methods yielding similar results.

GST-NOD320 was eluted from a glutathione column (Sigma, St. Louis, MO, or Amersham Pharmacia Biotech, Piscataway, NJ) with 20 mM glutathione in 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.9), 50 mM NaCl, 1 mM Na_2EGTA, 2 mM Mg acetate, and 100 μM ATP (0.4–2-fold molar ratio of ATP to GST-NOD in the peak fraction). The eluate was then dialyzed, centrifuged at 100,000 × *g*, and stored at 4°C. The concentration of NOD was determined by the Bio-Rad (Hercules, CA) protein assay with the use of bovine serum albumin as a standard. Motor concentrations were based on final protein concentrations rather than active site concentration.

Construction, Purification, and Motility Assays of Dm BCCP-Kinesin401 (K401B), K401B$_{(RFRP)}$, and K401B$_{(REAP)}$

Drosophila conventional kinesin (aa 1–401) fused to BCCP was generously provided by Jeff Gelles (Brandeis University, Waltham, MA) and was subcloned into pet28b and purified by established protocols. Site-directed mutagenesis of the adenine-binding site from RFRP to RFAP and REAP was done via the QuikChange kit (Stratagene), and the resulting constructs were sequenced on both strands. Motility assays were performed as outlined in Berliner *et al.* (1995) with the use of rhodamine-labeled tubulin, and rates were calculated on a calibrated screen by measurements of movements of microtubule ends.

Bovine Tubulin

Bovine tubulin was prepared as previously reported (Matthies *et al.*, 1993). Microtubules were prepared by adding 1 mM Mg GTP and 1 mM DTT to thawed microtubules and removing aggregates by a 100,000 × *g* spin in a TLA100.4 rotor. The supernatant was warmed to 37°C for 15 min and 5% dimethyl sulfoxide (0.1 mM phenylmeth-

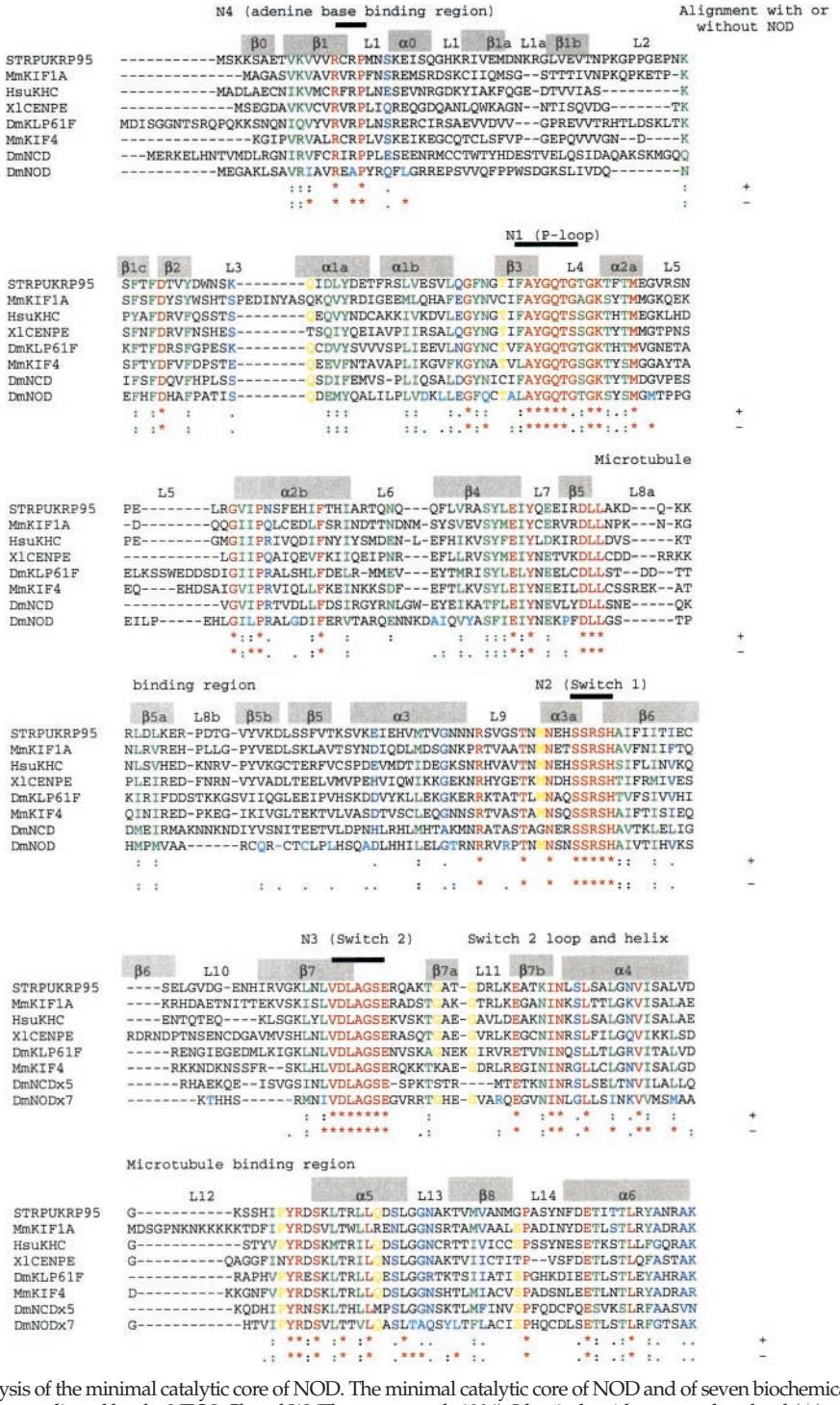


Figure 1. Sequence analysis of the minimal catalytic core of NOD. The minimal catalytic core of NOD and of seven biochemically characterized kinesins from different subfamilies were aligned by the NPS@ Clustal W (Thompson *et al*., 1994). Identical residues are colored red (★), strongly similar ones green (:) and weakly similar ones blue (.). The symbols for these levels of conservation are below the amino acids. Above the Hs uKHC sequence are the structural elements determined by x-ray crystallography (Kull *et al*., 1996; Sablin *et al*., 1996). Additionally, if four of the seven kinesins had an identical residue to NOD in a particular position, those residues were colored yellow. To highlight positions where NOD deviates from the most highly conserved residues, the other seven kinesins were aligned by the same program. The symbols for that lineup can be seen below the symbols for the lineup with all eight kinesins, including NOD. In positions where NOD is different, the symbol is colored by the color scheme used to indicate the level of conservation, i.e., if a residue is identical in the other seven kinesins, this is indicated by a red star (★), if a residue is strongly similar in the other seven but not in NOD, it is indicated by a green colon (:), and finally, deviations at a weakly similar position are indicated by blue periods (.). These positions are also denoted by coloring the appropriate residue in NOD light blue.

ylsulfonyl fluoride) was added for another 10 min. Taxol was added in increments (2 nM, 1 μM, 10 μM, and finally 80 μM). Microtubules were isolated by centrifugation at 40,000 $\times$ *g* for 30 min over a 40% sucrose cushion in BBR80 with 20 μM taxol.

ATPase Assays

ATPase activity was determined by measuring the rate of P_i formation by a continuous spectrophotometric assay coupled to purine nucleoside phosphorylase (Webb, 1992) (Molecular Probes, Eugene, OR) by measuring substrate accumulation at 360 nM in a temperature-controlled Cary 100 spectrophotometer at 25°C. All assays were done in 20 mM PIPES pH 6.9, containing 1 mM Na_2EGTA, 2 mM Mg acetate, 0.5 mM Na_2ATP, 1 mM DTT, 1 mg/ml bovine serum albumin (BSA), and 1 mM NaCl. Sufficient purine nucleoside phosphorylase (10 U/ml, and 250 μM substrate MESG [2-amino-6-mercapto-7-methylpurine riboside]) (Molecular Probes) was used to ensure that this enyzme activity was not rate limiting, and conditions were used such that <5% of the total ATP was used. Control studies demonstrated that GST-NOD320 with or without the PKA site had essentially identical ATPase levels. Phosphorylation by PKA for either version had no effect on ATPase activity. Purified glutathione (GST) lacked contaminating ATPases or MT-stimulated ATPases. Tubulin was varied in the range of 0.3–12 μM for the determination of the K_m (MT) in the presence of 0.5 mM MgATP, and MgATP was varied in the range of 25 μM-1 mM for the determination of the K_m (ATP) in the presence of 6 μM tubulin (a subsaturating concentration was used to minimize light-scattering effects). Mant-ATP was also tested under identical conditions. Experimental velocity data were plotted and curve fitted with DeltaGraph 4.5 (SPSS, Chicago, IL) by using user-defined parameters with the following equation: $V = (k_{cat}[S])/(K_m + [S])$ where V is initial ATPase rate, k_{cat} and K_m have the standard meaning, and [S] was either the concentration of ATP or tubulin. The curve-fit returned values for k_{cat} and the K_m for the varied "substrate." Experiments were repeated at least three times with independently isolated protein preparations and 12–25 points were collected for each data set.

Microtubule-binding Studies

GST-NOD320 was labeled with ^{32}P at the PKA phosphorylation site in linker region between GST and NOD by PKA (Promega, Madison, WI) in the presence of 40 μM ATP. Stoichiometry of phosphorylation ranged from 0.6 to 0.9. Control experiments indicated that GST-NOD320 with or without the PKA site had identical ATPase levels. Furthermore, treatment of GST-NOD320 with or without the PKA site had no effect on the basal ATPase or the microtubule-stimulated ATPase activity consistent with a lack of any PKA consensus sequence in the motor domain of NOD. These control experiments consisted of ATPase activity assays with the use of four microtubule concentrations and NOD treated with the following reagents: 1) PKA, 2) PKA plus 500 μM Walsh inhibitor (aa 5–24), and 3) 500 μM Walsh inhibitor. Walsh inhibitor is a potent PKA peptide inhibitor with a K_i of 3–5 nM (Walsh and Glass, 1991). These treatments had no effect (±5–10%) on the levels of NOD ATPase activity with any tubulin concentration. For the binding studies, 100 nM NOD was incubated in the presence of 300 nM to 100 μM microtubules in ATPase buffer except the final PIPES concentration was 32 mM rather than 20 mM, with the appropriately added phosphate analogs and nucleotides. Nucleotides and phosphate analogs were used at 1 or 2 mM, and $AlCl_3$ and $BeSO_4$ were supplemented with 5 mM NaF when appropriate. To mimic the ATP state, we used AMPPNP, ATP-γ-S, and ADP*BeF. ADP*BeF has been suggested to mimic ATP when bound to kinesin, but two myosin x-ray crystal structures of two different myosins with ADP*BeF have been interpreted as either an ATP or ADP*P state. For kinesin, it has been argued that ADP*BeF mimics bound unhydrolyzed ATP. ADP*phosphate transition states were generated by incubations with ADP and either AlF or vanadate. These mixtures were incubated for 1 h at room temperature, and microtubule-bound ^{32}P-GST-NOD320 was obtained by centrifugation for 10 min at 100,000 $\times$ *g* at 25°C. Pellets were resuspended in volumes equal to the supernatants and pellets, supernatants, and unspun total samples were analyzed by SDS-PAGE on 8% gels. GST-NOD320/MT binding was determined with the use of a Storm 820 imager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant (Molecular Dynamics) software; 0.04–0.15% pelleted or was bound to the centrifuge tubes and was subtracted from the remaining values. Depending on the experiments, 82–96% of the total labeled NOD pelleted with the microtubules with the various analogs. Dissociation constants were determined by fitting the data points to the Michaelis-Menten equation as described above. Experiments were done at least three times with independent preparations of NOD.

RESULTS

Sequence Analysis of NOD

The smallest portion of kinesin necessary for microtubule binding, ATP binding and hydrolysis, and force production is known as the minimal catalytic core. Sequence comparison of this portion of the kinesins has identified up to 10 subfamilies, with members of each subfamily having generally similar biological roles. Despite the observation that NOD is on chromosomes, it does not fall into the chromokinesin subfamily, but rather lies in an orphan category (Goldstein, 1993). Moreover, NOD lacks major structural elements found in virtually all kinesins, namely, the neck and the neck-interactor region.

The neck linker of kinesin is a region that interacts with the catalytic core and amplifies force production (Rice *et al.*, 1999; Case *et al.*, 2000). Sequence analysis indicates that NOD lacks a neck region (Vale and Fletterick, 1997) and also lacks the critical neck interacting residues (LGG) of the catalytic core as well (Figure 1, L13) (Sack *et al.*, 1997; Sablin *et al.*, 1998; Case *et al.*, 2000). The replacement of the more flexible GG with TA in NOD may hinder the nucleotide-dependent conformational changes observed in other kinesins.

Overall Sequence Comparison

NOD is one of the more divergent kinesins at the level of amino acid sequence (34% identical to the ubiquitous human kinesin heavy chain protein [(Hs uKHC]). Only 109 of the 318 amino acids of the minimal catalytic core of NOD are identical to Hs uKHC, and 72 are strongly similar as defined by the NPS@ Clustal W program (Thompson *et al.*, 1999). As shown in Figure 1, we compared NOD to seven kinesins from different subfamilies with demonstrated motile properties. The multiple alignment of those seven kinesins shows that they have 62 residues that are identical, 51 that are strongly similar, and 22 that are weakly similar. When NOD is then compared with these seven kinesins, 12 of the 62 fully conserved amino acids are changed, 6 of the 51 strongly similar amino acids are modified, and 10 of the 22 are no longer weakly similar.

When we focus on those 62 amino acids that are absolutely conserved in this set of seven motile kinesin proteins, we observe the most divergence in NOD around the adenine base-binding region (β1, L1, and α0), in regions surrounding the P-loop, in α4 (the switch I helix), and in one of the kinesin neck-linker interacting regions (L13) (Figure 1). The

Table 1. Critical MT-interacting residues identified in KHC (Woelhlke *et al.*, 1997): comparison to residues present in NOD

HsuKHC	NOD	SMY1[a]	COS2*	Region	Conservation
(A) 1.82–15.5 × wild-type K_mMT					
HsuKHC#					
R278	R	R	G	L12	Superfamily
K240	R	K	D	L11	Superfamily
Y274	V	S	N	L12	Superfamily
L248	R	Q	—[b]	L11	Superfamily
K256	L	Q	A	α4	Superfamily
K281	V	Q	T	α5	Superfamily
R284	T	E	T	α5	N-Terminal
(B) 0.6–1.62 × wild-type K_mMT					
HsuKHC#					
Y164	C	E	—	β5b	Superfamily
L153	M	L	N	β5a	N-Terminal
Q287	Q	K	K	α5	N-Terminal
E157	M	R	T	L8	Superfamily
P276	P	A	P	L12	Superfamily
Y138	Y	Y	C	L7	Superfamily
(C) 0.29–0.54 × wild-type K_mMT					
HsuKHC#					
E250	E	A	—	L11	Superfamily
E270	A	M	D	L12	N-Terminal
D279	D	E	Q	L12	N-Terminal
E170	C	P	E	β5	N-Terminal
E311	E	T	E	α6	Superfamily
D140	E	E	G	L7	Superfamily

[a] NOD, SMY1, or Cos2 were aligned against the seven motile kinesins of Figure 1 by Clustal W to identify the relevant residue.
[b] Indicates this position in the relevant kinesin is in a gap.

strongly conserved residues of these kinesins also diverge in NOD around the P-loop, in β4, in the β5/L8 microtubule region, in α3, in the L11 microtubule-binding region, and in L13 neck-linker interacting region. Aside from the deviations of conserved residues, NOD has deletions in two critical regions: L8b (microtubule binding), β6-L10 β7 (region linking switch I and II). These observations suggest that NOD may interact differently than other kinesins with both nucleotides and microtubules.

In Table 1 we compare the residues in Hs uKHC that are known to be critical for microtubule binding, to those residues found in the corresponding positions in NOD (Woehlke *et al.*, 1997). We only considered residues conserved in the kinesin superfamily or the N-terminal kinesins as indicated in Table 1 of Woehlke *et al.* (1997). The Hs uKHC residues were grouped into three categories based on their effect on the affinity of kinesin for microtubules: 1) those for which a change to alanine leads to a 1.8–15-fold increase in the K_m (MT) for microtubule-activated ATPase (class A); 2) those for which the change to alanine leads to a 0.6–1.2-fold effect on the K_m (MT) (class B); and 3) those for which the change to alanine results in a reduction in K_m (MT) to 0.29–0.6 of the wild-type level (class C). Five of the seven class A residues are positively charged amino acids. Of these, five charged residues, three are hydrophobic in NOD. Moreover, the lone hydrophobic residue in class A (L248) is instead positively charged in NOD, and the polar amino acid (Y274) is hydrophobic in NOD (V). When the class B amino acids are considered, polar residues are replaced with charged residues in NOD, and charged amino acids are replaced with hydrophobic or polar residues. The alteration of class C residues causes a pronounced reduction in the K_m for microtubules (Table 1, class C). These residues are generally negatively charged and are generally conserved in NOD (4/6); however, two of the six are not negatively charged.

In total, three critical residues for kinesin in L12/α5, one in L11, and three in β5 L8 are modified in NOD. Interestingly, triple mutants of kinesin from the first category in L12/α5 have no detectable motility (Woehlke *et al.*, 1997) and triple mutants in L11 are defective in the communication between microtubule binding and the active site, resulting in reduced velocity (0.27-fold wild-type rate) (Shimizu *et al.*, 2000).

The significance of the NOD deviations from the conserved sequence is borne out by the study of KHC mutants (Brendza *et al.*, 1999). For example, the *Drosophila* mutation of E164 (corresponding to E157 in the human sequence) to K reduces motility roughly fourfold, but reduces the k_{cat} only twofold. One of the class B residues was also identified in the above-mentioned genetic screen (Hs E270).

These differences in NOD sequence with respect to other well-studied kinesins raised questions as to the actual functional capacities of the NOD protein. Therefore, we set out to characterize a number of biochemical properties of this protein.

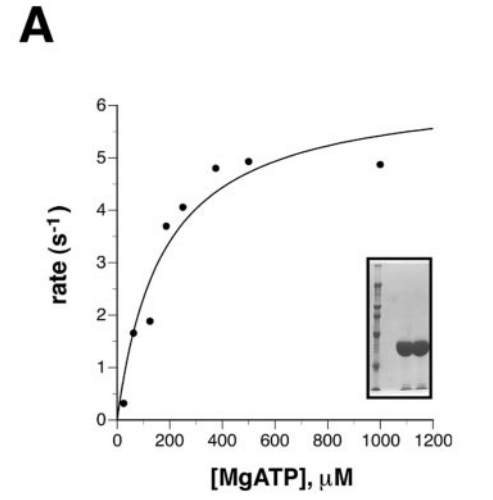
MT-stimulated ATPase Activity of Catalytic Core of NOD

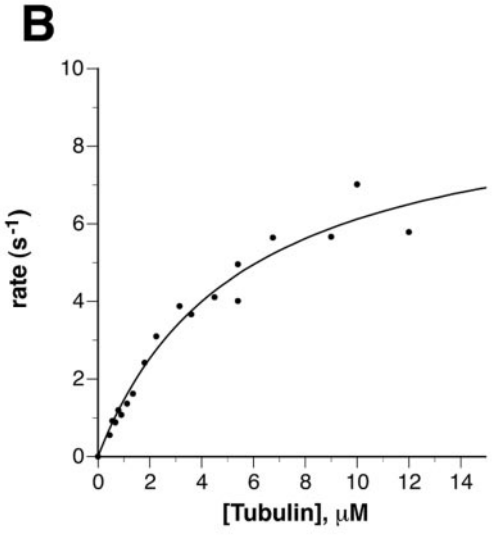
To examine the biochemical nature of the motor domain of NOD, we prepared an NOD-GST fusion protein consisting of amino acids 1–320 (Figure 2A, inset). This N-terminal portion of NOD is homologous to the minimal catalytic core for kinesin, which has been shown to contain the structural regions necessary for microtubule-stimulated ATP hydrolysis, and is a slow plus-ended motor (Case *et al.*, 2000). We first tested whether this NOD fusion protein could hydrolyze ATP, and if so, whether microtubules could stimulate its ATPase activity.

In the presence of 6 μM tubulin, the catalytic core of NOD is half maximally activated at 174 μM ATP [K_m(ATP) = 174 μM] (Figure 2A). In the absence of microtubules, NOD has very low ATPase activity. This ATPase activity can be increased >2000-fold by the addition of bovine MTs (Figure 2B and Table 2A). The microtubule-dependent activation of NOD ATPase activity has a strong ionic component. The concentration of polymerized tubulin dimer required for half maximal activation, K_M (MT), increases from 4.5 to ~11 μM by the addition of 50 mM NaCl. These results are consistent with the properties of the other kinesins studied and are summarized in Table 2A.

NOD Function Requires ATP Hydrolysis

NOD clearly hydrolyzes ATP in vitro and this ATPase is dramatically stimulated by microtubules. However, given that the SMY1A suppression of a myo2 defect in *Saccharomyces cerevisiae* does not require a functional P-loop, we





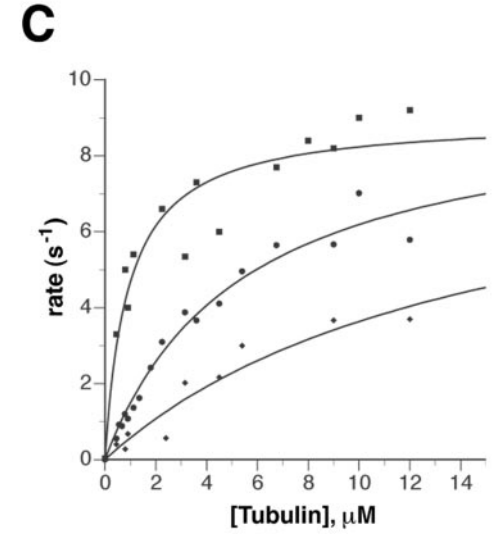


Table 2. Rate and dissociation constants of GST-NOD320

(A) Condition	k_{cat}, s^{-1}	K_m (MT), μM	K_m (ATP), μM
GST-NOD320	0.004		
MT + GST-NOD320	9.5 ± 0.5	5.5 ± 0.7	174
(B) Condition	**k_{cat}, s^{-1}**	**K_m (MT), μM**	**k_{cat}/K_M (MT)**
At 25°C			
MT + GST-NOD320	9.5 ± 0.5	5.5 ± 0.7	1.7
MT + GST-NOD320$_{DTW}$	9.0 ± 0.4	14.7 ± 1.8	0.6
MT + GST-NOD320$_{"DR2"}$	9.4 ± 0.7	1.1 ± 0.2	8.5
At 17°C			
MT + GST-NOD320	3.5 ± 0.3	7.1 ± 1.2	0.5
MT + GST-NOD320$_{"DR2"}$	3.2 ± 0.2	8.4 ± 1.1	0.4
(C) Condition	**K_d, μM**		
APYRASE	6.4 ± 0.5		
AMPPNP	4.5 ± 0.3		
ATP-γ-S	4.6 ± 0.4		
ADP-ALF	5.8 ± 0.5		
ADP-BeFL	3.3 ± 0.2		
ADP-VO_4	16.9 ± 1.9		
ADP	2.4 ± 0.2		

wondered whether the ATPase activity of NOD was essential for its function in vivo as well. A mutation in the P-loop of *nod* (*nod*$^{[DTW]}$) suggests that the function of NOD does require ATP hydrolysis. *nod*$^{[DTW]}$ displays a dominant meiotic phenotype that mimics that of recessive loss-of-function *nod* alleles, a cold-sensitive lethality, and an anaphase chromosome bridging cytological phenotype (Rasooly *et al.*, 1991, 1994). We decided to examine the biochemical consequences of this mutation.

The (*nod*$^{[DTW]}$) mutation is a change of the last serine of the P-loop to an asparagine. Based on comparisons to an analogous mutation in the yeast KAR3 protein (Meluh and Rose, 1990), this mutation has been assumed to generate a rigor complex between NOD and microtubules (Rasooly *et al.*, 1991, 1994). On the contrary, we found that the ATPase

Figure 2. Determination of the K_m (ATP), K_m (MT) for the minimal catalytic core of NOD (GST-NOD320): biochemical characterization of two NOD mutant proteins. (A) ATPase rate of 50 nM GST-NOD320 was measured in the presence of subsaturating levels of microtubules to determine the apparent K_m for ATP. These values were plotted by DeltaGraph and K_m values were obtained by curve fitting. The data was fit to the Michaelis-Menten kinetic model and the solid line was obtained from this curve-fitting procedure. The inset shows an SDS-PAGE gel showing molecular weight standards and the first two elutions of GST-NOD320. (B) ATPase rates of 50 nM GST-NOD320 were determined in the presence of various levels of polymerized tubulin and 0.5 mM ATP. The microtubule dimer concentration that led to half-maximum ATPase activation K_m (MT) and the k_{cat} were obtained by curve fitting with DeltaGraph. The solid line was obtained from the curve-fitting procedure (r = 0.97). (C) ATPase rates of various 50 nM GST-NOD fusions were measured and calculated as in B. Wild-type NOD-GST, NOD$_{DTW}$, and NOD$_{"DR2"}$ are represented by filled circles, diamonds, and squares, respectively. K_m (MT) and the k_{cat} were obtained by curve fitting with DeltaGraph with the following r values: GST-NOD320, 0.97; NOD$_{DTW}$, 0.92; and NOD$_{"DR2"}$, 0.96.

activity of NOD_{DTW}-GST320 can be activated to a similar extent as the wild-type protein; however, this activation requires threefold higher concentrations of microtubules [K_m (MT) = 15 μM] (Figure 2C and Table 2B). These results indicate that disruption of the coupling of the ATPase of NOD to the activation by microtubules does not allow NOD to function properly, and that whatever step in the chemical cycle of NOD is disrupted by the (*nod*$^{[DTW]}$) mutation can be overcome by higher concentrations of microtubules. Such a result is not expected if the mutation created an unreleasable rigor binding to microtubules.

NOD Also May Be Activated by Microtubules In Vivo

Several second-site intragenic suppressors of the dominant meiotic and cold-sensitive mitotic phenotypes of *nod*$^{[DTW]}$ have been characterized genetically and molecularly (Rasooly *et al.*, 1991, 1994). One of these (DR2) results from a change of a highly conserved aspartate to an arginine, at a site within or very close to a microtubule-binding region (Woehlke *et al.*, 1997) (homologous to Hs uKHC D_{144} in β5; Figure 1). Although this second mutation does not restore wild-type function to the NOD_{DTW} protein, it does eliminate the poisonous effects of the *nod*$^{[DTW]}$ P-loop mutation. Thus, we thought that studying this point mutation might be useful for testing the hypothesis that microtubules can activate the ATPase activity of NOD.

We call the protein bearing this single intragenic suppressor mutation $NOD_{"DR2"}$ to distinguish it from the double mutation *nod*^{DR2}, which in fact is comprised of two point mutations: the original *nod*$^{[DTW]}$ mutation in the P-loop and the intragenic suppressor mutation in a potential microtubule-binding region. Analysis of the protein $NOD_{"DR2"}$ demonstrates that this mutation leads to a dramatic reduction in the concentration of microtubules required for the allosteric activation of ATPase activity [K_m (MT) = 1.1 μM; Figure 2C and Table 2B].

Given that microtubule concentrations in vivo should be similar in wild type and mutant spindles, one could imagine then that the intragenic suppressor mutation in the microtubule-binding region partially suppresses portions of the defects of the *nod*$^{[DTW]}$ mutation by increasing ATPase rates at equivalent microtubule concentrations. Further kinetic studies of NOD_{DTW}, $NOD_{"DR2"}$ and the double mutant will indicate which step is slower in NOD_{DTW} and which step is accelerated by the mutation in $NOD_{"DR2"}$. Different steps of the mechanochemical cycle may be modified and this could be the reason why only portions of the *nod*$^{[DTW]}$defects are corrected by the *nod*$_{DR2}$ mutation. However, the analysis of $NOD_{"DR2"}$ indicates that NOD interacts and is activated by microtubules in vivo.

The catalytic efficiency of kinesins can be estimated by the ratio k_{cat}/K_m(MT) (Brendza *et al.*, 1999). By this criterion, NOD_{DTW} and the intragenic suppressor $NOD_{"DR2"}$ have one-third lower and fivefold higher, respectively, catalytic efficiency than the wild-type protein (Table 2B). The catalytic efficiency of $NOD_{"DR2"}$ is more strongly suppressed than the wild-type protein at 17 versus 25°C (Table 2B), suggesting that this region activates the ATPase via an energy-dependent conformational change. In the $NOD_{"DR2"}$ protein, microtubule binding and activation of ATPase activity are more efficiently coupled, but this is reversed at a lower temperature.

Energy input may be involved in the allosteric effects of microtubule binding on kinesins. Microtubule binding must induce a conformational change(s) that causes ADP release to be accelerated >1000-fold (Hackney, 1988) and the hydrolytic step to be increased 10-fold (Ma and Taylor, 1995a,b). The temperature dependence of the effect of $NOD_{"DR2"}$ mutant point to an energy requirement of this conformational change. Finally, these results suggest that NOD can be activated by microtubules in vivo.

ADP Is Tightly Bound by Purified GST-NOD320

For all other kinesins studied so far, ADP release is the rate-limiting step for the ATPase cycle (Hackney, 1996). Indeed, purified kinesins are recovered in the ADP-bound state. To determine whether this is also the case for NOD, we incubated NOD bound to a glutathione column with either α- or γ-labeled ATP. NOD was eluted from the column with glutathione, and we observed that the fractions containing NOD coeluted with α-labeled, but not γ-labeled nucleotide. The hydrolyzed phosphate does not stay bound to NOD, suggesting that as is typical for kinesins, ADP binding is tight and phosphate release is rapid. These data, in combination with the demonstration that microtubules activate the ATPase activity of NOD, suggest that the interaction of NOD with microtubules leads to a conformational change that greatly accelerates ADP release (basal ATPase rate is 0.004/s, which is stimulated by microtubules to 9.4/s), as is the case for other kinesins (reviewed in Hackney, 1996).

NOD Does not Generate Microtubule Gliding In Vitro

The catalytic core of NOD displays some properties of a typical kinesin, so we set out to test whether NOD has force-producing capabilities by using microtubule gliding assays. We attempted to demonstrate the ability of NOD to produce microtubule gliding by coating glass with GST antibodies and allowing full-length GST-NOD666 to bind to the glass, thereby minimizing the direct interaction of NOD with the bare glass surface. This method has been previously used successfully for a number of kinesin-GST-fusions (Stewart *et al.*, 1993; Boleti *et al.*, 1996). Although we did observe microtubule binding to the coverslip, we found no evidence for microtubule gliding in four independently isolated preparations (our unpublished data). To enrich for productive NOD and microtubule interactions, we also allowed GST-NOD to bind to microtubules first then perfused this mixture into the motility chamber, but again observed only microtubule binding (three independent preparations).

Because the highly basic NOD protein could still potentially be inactivated by the glass surface, we used positively charged streptavidin-coated coverslips to test whether a 6X-His-tagged truncated version of NOD (NOD485B) can generate microtubule gliding. NOD485B lacks the cargo-binding domain, thereby avoiding any difficulty of copurifying DNA and contains an in vivo biotinylated C terminus. Similarly modified kinesins have been shown to generate microtubule gliding over streptavidin bound to biotinylated-BSA which was, in turn,

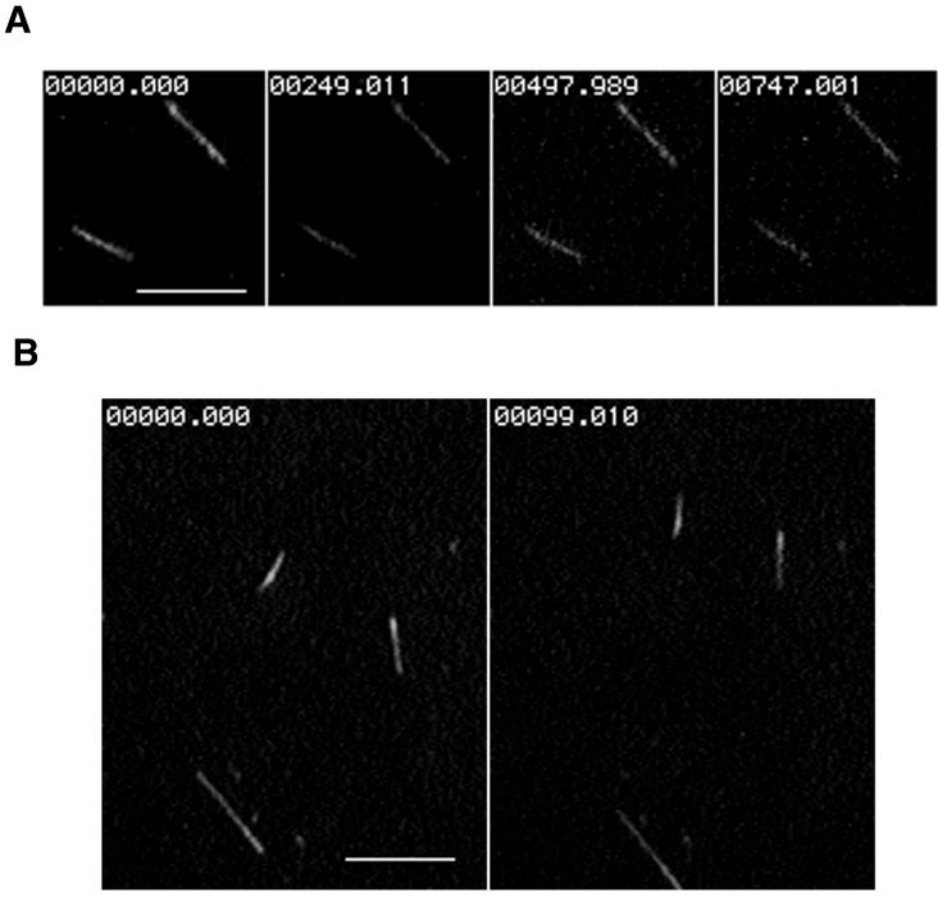


Figure 3. (A) NOD-485B does not generate microtubule gliding in vitro. In vivo biotinylated NOD-485B was perfused into chambers coated with protein layers consisting of streptavidin bound to glass coated with biotinylated BSA. (B) *Drosophila* K401B was tested with the use of identical conditions.

bound to the coverslip (Berliner *et al.*, 1994; Gheber *et al.*, 1999). It is therefore not likely that this modification should interfere with the potential microtubule-gliding activity of NOD485B. As shown in Figure 3, when we used these streptavidin-coated coverslips, we noted microtubule binding only (three independent preparations), despite demonstrable microtubule-stimulated ATPase activity of NOD485B (Table 3). If the surface was treated with 5 mM biotin before the application of NOD485B, substantially fewer microtubules were bound (our unpublished data), indicating that NOD is responsible for the binding. These results argue that NOD does not possess motile properties in vitro.

NOD Binding to MTs Is Strongest in Presence of ADP

Given the seemingly contradictory observations that NOD has microtubule-stimulated ATPase, yet fails to display motility, we set out to further characterize the biochemical properties of this protein. We next tested whether NOD has the nucleotide hydrolytic state-sensitive microtubule binding, which is critical for force production in typical kinesins (Crevel *et al.*, 1996). Members of several subfamilies of the kinesin superfamily have been shown to display transitions from strong to weak affinities for microtubules, dependent upon the hydrolysis state of the bound nucleotide (Crevel *et al.*, 1996; Lockhart and Cross, 1996). These different binding states are critical for the various conformational states required for unidirectional transport. To determine whether the affinity of NOD for microtubules also depends on the hydrolysis state of the bound nucleotide, we used several

Table 3. Mutational analysis of adenine binding site: uncoupling of ATPase activity and microtubule gliding in vitro

Condition	k_{cat} s^{-1}	K_m (MT) μM	MANT-ATP/ATP %[a]	Motility rate $nm \cdot s^{-1}$
GST-K333	29.8	0.24		
GST-K333$_{RFAP}$	10.2	0.66		
GST-K333$_{REAP}$	2.8	0.72		
K401B	17.3	0.2	100	670
K401B$_{RFAP}$	11.7	1.6	81	1.3
K401B$_{REAP}$	2.6	1.8	50	N.D.
NOD485B	22	0.5	0.8	N.D.

N.D., none detected.

[a] Phosphate release was measured in the presence of either 0.2 mM MANT-ATP or 0.2 mM ATP. Shown is the ratio of phosphate release in the presence of MANT-ATP versus ATP.

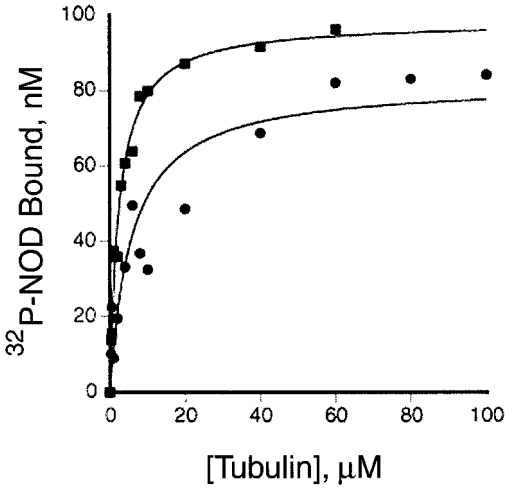


Figure 4. Determination of the binding affinity of NOD for microtubules in the presence of ADP or AMPPNP. ^{32}P-Labeled NOD (100 nM) was incubated for 1 h with various levels of polymerized tubulin in the presence of AMPPNP (●) or ADP (■), sedimented by centrifugation, and the amount of bound NOD was measured by analyzing SDS-PAGE gels with the use of a PhosphorImager. The number of nanomoles of NOD that sedimented with the microtubules was calculated and plotted by DeltaGraph versus the levels of polymerized tubulin. K_d values were obtained by curve fitting and the r values were the following: AMPPNP, 0.91, and ADP, 0.96.

ATP and phosphate analogs in microtubule sedimentation studies.

In the presence of microtubules, ATP is rapidly hydrolyzed by kinesins and by NOD. Therefore, typically ATP γ-S, AMPPNP, and ADP*BeF are used to mimic unhydrolyzed ATP, whereas a combination of ADP with either AlF or vanadate mimics the transition states of ADP and bound phosphate. Nucleotide-free states of kinesins can be generated by treatment with the enzyme apyrase, which removes nucleotides by degrading ATP to AMP.

An example of one of these binding studies is shown in Figure 4 and all binding experiments are summarized in Table 2C. Unlike other kinesins of the various subfamilies previously characterized, we found that for NOD it is ADP, rather than the nonhydrolyzable ATP analogs, that leads to the tightest microtubule-binding state (K_d = 2 μM). This binding constant in the presence of ADP is roughly 3 times tighter for NOD than is observed for a comparable truncated version of conventional kinesin (Hs K322-ran17-GFP, K_d = 6.9 μM; Case *et al.*, 2000). All of the ATP analogs tested lead to weaker binding states than in the presence of ADP. For example, if one considers ADP*BeF to be an ATP rather than an ADP*P analog (see MATERIALS AND METHODS) then NOD*ADP (K_d = 2 μM) and NOD*ATP (NOD*ADP*BeF, K_d = 3.3 μM) states have, at best, very similar affinities for microtubules. In the ATP state, NOD appears to bind more weakly than do other kinesins. In comparison, in the presence of AMPPNP, GST-NOD320 (K_d = 6.4 μM) binds with 10-fold lower affinity than a comparably truncated conventional kinesin (Hs K322-ran17-GFP, K_d = 0.7 μM; Case *et al.*, 2000).

NOD was also prepared in the nucleotide-free state. However, unlike other kinesins, where the nucleotide-free state leads to the tightest microtubule/motor interaction, nucleotide-free NOD has a low-affinity microtubule-binding state (6.4 μM) relative to the ADP*NOD state, and to the nucleotide-free state of other kinesins.

Various ADP*phosphate transition states have been suggested to exist for kinesins, and the binding seen with ADP*ALF and ADP*vanadate may represent these states. As has been observed for other kinesins, ADP*vanadate generates the weakest binding state for NOD (K_d = 16.9 μM), but ADP*F also generates a low-affinity binding state (K_d = 5.8 μM) (Table 2C). These results minimally show that NOD, just as several other kinesins, can assume a low-affinity microtubule-binding state in the presence of vanadate.

In summary, the results of these binding studies suggest that nucleotides do alter the affinity of NOD for microtubules; however, unlike all other kinesins studied, neither the nucleotide-free nor the ATP condition lead to the strongest binding state. NOD binds to MTs with a lower affinity than other kinesins in the nucleotide-free and ATP states, but with a higher affinity in the ADP-bound state. Assuming NOD has a similar basic mechanochemical cycle as other kinesins, these results argue that NOD is not likely to use the energy stored in ATP to produce vectorial transport, because ATP binding to NOD does not lead to a tightly bound NOD/microtubule interaction required for unidirectional transport. If the ATP state were capable of producing force then the ADP and nucleotide states would generate a counteracting drag, because the ADP, nucleotide-free, and ATP states generate similar affinities for microtubules.

ATP-Analog Studies Suggest Important Differences in ATP-binding Site

NOD has been shown above to differ from the other well-characterized kinesins at several highly conserved residues, specifically those surrounding the nucleotide-binding regions. We wondered whether these amino acid substitutions might actually alter the properties of the ATP-binding site. To test this idea, we began with a modified nucleotide, shown previously to be a good substrate for kinesin (Ma and Taylor, 1995; Moyer *et al.*, 1998), to probe the conformation of the nucleotide-binding pocket of NOD. Kinesin, myosins, and G proteins hydrolyze nucleotides that have been modified at either the 2 or 3 positions with *N*-methyl-anthronyl adenosine triphosphate (MANT). In contrast, we found that MANT-ATP appears to be a very poor substrate for NOD. With the use of 6 μM MT and 0.5 mM ATP the k_{cat} is 4.9 s^{-1}, whereas with the same microtubule concentration, but with 0.5 mM MANT ATP, the k_{cat} is 0.8 s^{-1}. This result was surprising because 1) typically kinesins can use MANT-ATP almost as well as unmodified ATP; and 2) the MANT modification is at a position of the ribose ring of ATP, which is solvent exposed based on all crystallized kinesins. This study indicates that the nucleotide pocket of NOD is different than that of other kinesins and could contribute to the uncoupling of ATPase and potential transport activity.

Conserved Residues for Adenine Binding That Are Required for Efficient Coupling of ATP Hydrolysis and Motility Are Altered in NOD

To determine the differences in the manner in which NOD handles MANT-ATP, we more closely compared the nucle-

otide-binding regions of NOD with those of other well-characterized kinesins. In other kinesins, the adenine-binding site is composed of a highly conserved RxRP motif (Figure 1, N4 motif); the variable X appears to be subfamily specific, and generally hydrophobic. In contrast, NODs sequence in this region is REAP; it lacks the conserved arginine found in 104/106 kinesins. Four other kinesins lack this conserved arginine: *S. cerevisiae* Kip2 (Kip 2/3 subfamily), Smy1 (orphan subfamily), *Drosophila* Cos2 (orphan family), and *Caenorhabditis elegans* CeMO3d4.1b (MKLP subfamily).

Of all kinesins studied to date, only NOD and these four other kinesins lack the second arginine in this site (RXRP). Moreover, all five of these divergent kinesins lack the conserved glutamate located six amino acids C-terminal to the position where the second conserved arginine should be. This glutamate has been found to stabilize the second arginine via a salt bridge (Kull *et al.*, 1996; Sablin *et al.*, 1996; Sack *et al.*, 1997). Moreover, the microtubule-depolymerizing I-kins, at least some of which also lack demonstrable in vitro motility (Desai *et al.*, 1999), have a charged residue in the X position, as does NOD.

We therefore modified this RXRP sequence in *Drosophila* kinesin heavy chain (K401B) to see whether this sequence plays a role in motility. Wild-type K401B, fused to the in vivo biotinylated peptide BCCP, has previously been shown to translocate microtubules at wild-type rates (Berliner *et al.*, 1994). K401B translocated microtubules at 670 nm/s, whereas K401B with RXRP mutated to RFAP translocated at only 1.3 nM/s (Table 3). The doubly modified kinesin (REAP) did not demonstrate motile properties at all (three preparations). Despite reduced motility, all of these proteins in both monomeric and dimeric forms showed microtubule-stimulated ATPase activity (Table 3, K_{cats} for $K401B_{WT}$ = 17.3, $K401B_{RFAP}$ = 11.7, and $K401B_{REAP}$ = 2.6/s, respectively).

Comparing the ATPase rates in the presence 0.2 mM ATP to the rate observed with the use of 0.2 mM MANT-ATP, the RFAP K401B single mutant showed a 10% reduction in the k_{cat}, the REAP double mutant showed a 50% reduction, and wild-type NOD485B showed a 92% reduction in activity (Table 3). These results suggest that part of the reason NOD uses MANT-ATP more poorly than unmodified ATP is a result of the binding properties of the adenine base-binding motif.

In summary, these results argue that the modified adenine binding of NOD serves to uncouple ATP hydrolysis from force production. These results also point to the importance of the orientation of ATP in the binding site for the motility of kinesins, but because NOD has so many other changes in critical residues, we did not attempt to replace NODs adenine-binding site with that of kinesin heavy chain.

DISCUSSION

The NOD protein is similar to other kinesins in several fundamental ways. NOD has microtubule-stimulated ATPase activity, and the affinity of NOD for microtubules can be modified by the state of the bound nucleotide. ATPase activity is stimulated by microtubules in a salt-dependent manner, and ADP release may be the rate-limiting step in the ATPase cycle. However, in perhaps more fundamental ways, the NOD protein is very different from the rest of the kinesin superfamily. ATP binding to NOD does not lead to the tight microtubule-binding state found in other kinesins. Microtubule-binding affinities do not differ significantly in the presence of ATP, ADP, or in the absence of nucleotide, which suggests a different function for NOD than for those found in the other members of the kinesin family.

Despite the many differences in the sequence of NOD to kinesins, NOD demonstrates substantial activation by microtubules. This is surprising considering some of the differences in sequence of NOD and motile kinesins. One critical difference is that NOD has a relatively low microtubule affinity in the presence of ATP, but fairly tight binding in the presence of ADP. Certain residues of NOD that are different than those found to be critical for Hs uKHC may generate the relatively weak binding in the presence of ATP, and others may lead to the relatively tight binding in the presence of ADP. The other possibility is that ATP binding, which normally leads to a tight microtubule-binding state, is not communicated to the microtubule-binding site of NOD.

NOD Is Unlikely to Function as a Motor

Observations that the unidirectional transport generated by kinesins and myosins depend upon transitions from strong to weak binding states relative to their respective "track" lead us to suggest that NOD may not be capable of producing a vectorial powerstroke. NOD does not appear to undergo the conventional strong-to-weak microtubule-binding state transition. Microtubule affinities in the ADP state and nucleotide-free states of NOD, relative to their affinity in the ATP state, would limit the potential force production in the presence of ATP due to "drag" created by these states. We conclude that NOD has evolved to become a kinesin that is no longer likely to produce unidirectional transport in a conventional sense.

Clark *et al.* (1997) suggested that NOD may be a minus-end–directed motor. Their study used a construct that substituted the motor domain of Kin:β-gal with the putative motor domain of NOD. In cells with defined microtubule polarity, the NOD:kin:β-gal fusion protein appeared to function as a minus end reporter for microtubules. NOD:kin:β-gal localized to the apical cytoplasm in epithelial cells, and to the poles of mitotic spindles in dividing cells, suggesting that the head of NOD may be a minus-end–directed motor. In a different study, expression of the minimal catalytic core of NOD in isolation led to the accumulation of NOD along the entire length of the microtubule arrays (Afshar *et al.*, 1995b), not predominantly at the poles as is the case for the NOD:kin:β-gal fusion.

Several lines of evidence suggest that the result obtained by Afshar *et al.* (1995b) is more representative of the native NOD. First, as reported here, the minimal catalytic core of NOD does not have the appropriate properties to suggest minus-end–directed motility. Second, XKCM1 which, like NOD, also does not appear to have motile properties, does label microtubule ends in a manner similar to that observed for NOD:kin:β-gal by Clark (1997) and Desai *et al.* (1999). Finally, the NOD:kin:β-gal fusion consisted of the minimal catalytic core of NOD, plus two additional amino acids, fused to the neck linker of *Drosophila* kinesin (i.e., a chimeric neck). The remainder of the protein consists of a portion of the stalk of kinesin linked to the β-gal enzyme. Kinesin would likely dimerize with this NOD:Kin:β-gal fusion because kinesin is expressed in large excess, has a high affinity

for dimerizing, and the NOD:kin fusion contains the regions required for kinesin dimerization. Furthermore, this NOD:kin:β-gal/kinesin heterodimer may also bind kinesin light chains. The biochemical properties of this protein complex would be hard to predict.

Model for NOD Function in Meiosis

In the absence of NOD, nonexchange chromosomes fail to segregate properly. Although cytologically fairly normal spindles develop, the nonexchange chromosomes leave the spindle at high frequencies, and occasionally both homologs of nonexchange chromosomes are found on the same side of the spindle (Theurkauf and Hawley, 1992). Immunolocalization of NOD indicates that NOD is found along the entire length of all chromosomes (Afshar *et al.*, 1995a,b). These results indicate that NOD is essential for the segregation of nonexchange chromosomes by acting on the arms of chromosomes. In the absence of NOD, the achiasmate chromosomes tend to leave the spindle, but other motors or proteins must still allow microtubules to associate with chromatin. Spindles have been shown to form around both the main chromosomal mass and around the chromosomes that have escaped from the spindle.

We propose that NOD cross-links microtubules to the chromosomes; therefore, these chromosomes tend to be stretched toward opposite sides of the spindle as the spindle elongates during spindle assembly. Several hypotheses can explain how this role of NOD would result in the phenotype of *nod* null alleles. One possibility is that as the chromosomes stretch, NOD has more binding sites along the entire chromosome, and the cross-linking activity of NOD regulates microtubule dynamics. NOD would act as a stabilizing microtubule-associated protein held along the microtubules by the interaction with both chromosomes and microtubules. Stabilizing microtubules around the chromosomes could slow poleward flux, allowing other *Drosophila* chromosome associated plus-end–directed motors (KLP38B and potentially KLP31E) to maintain chromosomes at the metaphase plate. In the absence of NOD, poleward flux is elevated, allowing chromosomes to move to the end of the short microtubules where they tend to dissociate. It has been shown that the *Drosophila* meiotic spindle consists of many short microtubules, most of which do not actually extend to the poles (Theurkauf and Hawley, 1992).

A second, but not mutually exclusive, hypothesis is that the cross-linking activity of NOD could regulate the tension on chromosomes. This tension could regulate other kinesins' motor activity that is responsible for the poleward motion of the chromosomes. The cross-linking activity of NOD may be dynamic. For example, based on our microtubule-binding studies, binding may be weaker in the nucleotide-free state, and NOD may be released from microtubules in this state. These transient attachments could regulate the tension on the chromosomes. In the nucleotide-free state, one would expect the chromosome to relax to the lowest stretched state, because NOD has a low affinity for microtubules in this state. This cycle in microtubule affinity would allow the chromosome to stretch maximally and distribute the tension evenly until the metaphase position is attained.

Chromatin stretching has been observed during *Drosophila* female meiosis (Theurkauf and Hawley, 1992; Page and Orr-Weaver, 1997; Matthies *et al.*, 1999). At the onset of *Drosophila* female meiosis, chromatin exists as a compact sphere of 4–5 μm, which is capable of elongating into a structure of up to 20 μm in length (Page and Orr-Weaver, 1997; Matthies *et al.*, 1999) in a microtubule-dependent manner (Page and Orr-Weaver, 1997). This stretching of chromatin may be due in part to the action of microtubule-based motors. The force production abilities and properties of both kinesin (Visscher *et al.*, 1999) and dynein (Gross *et al.*, 2000) have been shown to be modified by load, and dynein has been proposed to have a role in centromere tension leading to metaphase arrest and in chromosome motility (Starr *et al.*, 1998; Sharp *et al.*, 2000).

How other motors, which play roles in chromosome dynamics, respond to loads is unknown. It would be reasonable to propose that the cross-linking of chromatin to microtubules by NOD could, in turn, regulate the activity of microtubule-based motors on the chromosomes by modifying the load on them. Perhaps the poleward forces are more strongly affected by tension, and therefore plateward positioning of chromosomes is favored by NOD. Such an activity would be consistent with an observation made by the Salmon lab, which led them to the conclusion that poleward forces are slowed by a "brake," or governor, rather than an active antipoleward force (Skibbens *et al.*, 1995). The analyses of $nod^{[DTW]}$ flies, and our biochemical analysis of this mutation and one of the partial revertants, could substantiate this view. Cytological analysis of $nod^{[DTW]}$ and the revertants show that $nod^{[DTW]}$ leads to mitotic anaphase bridging, and this phenotype is reverted by the partial revertants (Rasooloy *et al.*, 1991). One explanation of the anaphase bridging is that NOD_{DTW} is more poorly activated by microtubules, and therefore poleward forces are not constrained. This leads to rapid anaphase chromosome movement, and because sister arm cohesion lags behind the centromere cohesion release, there may not be sufficient time to allow sister cohesion to be released.

It has been observed that kinetochores, which reverse their poleward movement in anaphase and therefore are presumably under less tension from forces on the arms, reinitiate poleward movement at much higher rates until they catch up with the other kinetochores (Skibbens *et al.*, 1995; Shelby *et al.*, 1996). In oocytes lacking NOD, the chromosomes that lack chiasmata leave the spindle at rates approaching 70%. If other motors or chromatin/microtubule cross-linkers function on these chromosomes, they do so very poorly. In that case, one could argue that NOD may make these other motors more "processive," allowing the chromosomes to be maintained at the metaphase plate. In conclusion, we propose that NOD dynamically cross-links chromatin and microtubules, which leads to regulation of tension across the centromeres and thereby regulates the activity of motors responsible for the poleward migration of chromosomes.

A third hypothesis is that NOD functions to mediate homolog cohesion via its two DNA-binding domains and this cohesion is regulated by the microtubule-activated ATPase activity. Such a NOD-based cross-linking could help maintain metaphase arrest, and this would be particularly critical for chromosomes lacking chiasmata. NOD could potentially dimerize via the HMG14/17 domain upon nucleosome binding as has been demonstrated for the HMG14/17 protein. This dimerization would generate many potential

chromosome-binding sites for cross-linking. Then, in a cell cycle-dependent manner, conformational changes in the motor domain occur due to microtubule-activated ATPase activity, thereby releasing cargo. This would allow the release of cohesion between chromosomes.

Are There Other Kinesins Like NOD?

There are three other kinesins that lack this conserved arginine from the RXRP motif: *S. cerevisiae* Kip2 (Kip 2/3 subfamily), SMY1 (orphan subfamily), Cos2 (orphan subfamily), and *C. elegans* CeMO3d4.1b (MKLP subfamily). SMY1 also lacks certain critical residues defined by the scanning alanine mutagenesis (Woehlke *et al.*, 1997). SMY1 has been suggested to no longer function as a microtubule-based motor (Lillie and Brown, 1998). Overexpression of SMY1 partially corrects for the defects due to the absence of Myo2p, an *S. cerevisiae* myosin (Lillie and Brown, 1992). However, this rescue does not depend on microtubules, nor on critical residues in the P-loop of SMY1. SMY1 localizes to a region lacking microtubules (Lillie and Brown, 1994) and the removal of microtubules has no effect on the SMY1 localization pattern nor on SMY1 cap formation (Lillie and Brown, 1998). However, because microtubules are not essential for the yeast secretory pathway, one cannot rule out that SMY1 may have some microtubule-dependent functions in the secretory pathway. Another member of the orphan subfamily, COS2, binds to microtubules in an ATP-insensitive manner. The proposed function of this kinesin is to sequester components of a signaling complex in the cytoplasm away from the nucleus. This interaction is regulated by the initial signal of this cascade (Hedgehog; Robinson *et al.*, 1997). This model also does not assign traditional motor activities to Cos2.

The I-kins, at least some of which also lack demonstrable in vitro motility, have a charged residue in the X position, as does NOD. Interestingly, a *C. elegans* chomokinesin (CeT01G1.b) has a modified RXRP motif (SIRP) and may have similar biochemical properties to NOD, because mutating the first arginine to either lysine or alanine leads to a substantial reduction of motility in kinesin heavy chain (Kapoor and Mitchison, 1999). Seven other kinesins are known that lack the last conserved proline from this motif, and three of these seven also lack the conserved glutamate that stabilizes the second arginine of this motif. Kinesins lacking this proline are a *C. elegans* C-terminal kinesin (CeW02B12.7), *S. cerevisiae* Kip3 (Kip2/3 family), and five members of the BimC family (*Aspergillus nidulans* BimC, *Aspergillus thaliana* ATFC1a, *Schizosaccharomyces pombe* Cut7, and *S. cerevisiae* Cin8 and Kip1).

BimC proteins do function as very slow motors, perhaps as a consequence of a 10-fold reduction in the activation of ADP release (Lockhart and Cross, 1996). However, recent studies of the CIN8 protein suggest that its capacity for microtubule bundling is substantially more critical to its biological function in spindle maintenance than is its capacity for microtubule-based motility (Gheber *et al.*, 1999). Thus, it is possible that other kinesin superfamily members besides NOD may function primarily not as traditional motors but rather as cross-linkers of cargoes to microtubules (Goldstein and Philp, 1999).

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