**Drosophila** Nod Protein Binds Preferentially to the Plus Ends of Microtubules and Promotes Microtubule Polymerization In Vitro

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Nod, a nonmotile kinesinlike protein, plays a critical role in segregating achiasmatic chromosomes during female meiosis. In addition to localizing to oocyte microtubules, we show that functional full-length Nod-GFP (Nod<sub>FL</sub>-GFP) localizes to the posterior pole of the oocyte at stages 9–10A, as does kinesin heavy chain (KHC), a plus end-directed motor. This posterior localization is abolished in grk mutants that no longer maintain the microtubule (MT) gradient in the oocyte. To test the hypothesis that Nod binds to the plus ends of MTs, we expressed and purified both full-length Nod (Nod<sub>FL</sub>) and a truncated form of Nod containing only the motorlike domain (Nod<sub>318</sub>) from *Escherichia coli* and assessed their interactions with MTs in vitro. Both Nod<sub>FL</sub> and Nod<sub>318</sub> demonstrate preferential binding to the ends of the MTs, displaying a strong preference for binding to the plus ends. When Nod<sub>318</sub>-GFP:MT collision complexes were trapped by glutaraldehyde fixation, the preference for binding to plus ends versus minus ends was 17:1. Nod<sub>FL</sub> and Nod<sub>318</sub> also promote MT polymerization in vitro in a time-dependent manner. The observation that Nod is preferentially localized to the plus ends of MTs and stimulates MT polymerization suggests a mechanism for its function.

**INTRODUCTION**

In *Drosophila melanogaster* female meiosis, the controlled movement of achiasmatic chromosomes on the meiotic spindle is dependent on Nod, a 666 amino acid kinesinlike protein that localizes along the arms of meiotic chromosomes (Zhang et al., 1990; Alshar et al., 1995a, 1995b; see Figure 1B). In the absence of functional Nod protein, achiasmatic chromosomes dissociate from the main chromosome mass immediately after nuclear envelope breakdown (NEB) by migrating off the ends of the developing spindle (Theurkauf and Hawley, 1992). Both genetic and cytological studies suggest that Nod functions to hold chromosomes at or near the metaphase plate, opposing the poleward force exerted by the kinetochores (Theurkauf and Hawley, 1992; Matthies et al., 1999). Given that the microtubules (MTs) in the oocyte spindle are arranged with their plus ends at or near the metaphase plate (Riparbelli and Callaini, 2005), these results initially suggested that Nod acted as a plus end-directed motor that pushes chromosomes toward the metaphase plate (Matthies et al., 1999). A function of Nod in pushing chromosomes arms toward the metaphase plate has also been demonstrated in *Drosophila* mitotic cells by Goshima and Vale (2003). These authors have shown that in cells in which Nod function is ablated by RNAi, the arms of most chromosomes were extended along the spindle axis toward one of the two poles rather than being held at or near the metaphase plate.

However, several lines of evidence show that the motorlike domain of Nod lacks the capacity for vectorial transport (Matthies et al., 2001). First, Nod lacks three major structural elements that are found in virtually all kinesins (the neck, the neck-interactor region, and a crucial salt bridge) and are required for movement (Rice et al., 1999; Case et al., 2000). Second, although the motorlike domain of Nod is 34% identical to the prototypical human kinesin heavy chain protein (Hs KHC) motor domain, when one considers only residues that are strongly conserved within the KHC superfamily, 12 of the 62 fully conserved amino acids are changed in Nod, and 6 of the 51 strongly similar amino acids are different in Nod (Matthies et al., 2001). Similarly, with respect to the 10 residues in Hs KHC that are most critical for MT binding (Woehlke et al., 1997), 7 of these amino acids are altered in Nod. Third, although MTs activate the ATPase activity of Nod more than 2000-fold (Matthies et al., 2001), this ATPase activity is not coupled to vectorial movement (i.e., Nod does not produce MT gliding in vitro; Matthies et al., 2001). Fourth, the substitution of a single amino acid in the ATP-binding motif of *Drosophila* KHC with the analogous amino acid from Nod results in a drastic inhibition of motility.

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Abbreviations used: MT, microtubule; GFP, green fluorescent protein; KHC, kinesin heavy chain; NEB, nuclear envelope breakdown.
Figure 1. The structure of the Drosophila Nod kinesin-like protein. (A) A schematic comparison of the structure of the Nod\(_{d1}\)-GFP protein expressed for these studies and the Nod-KHC-βgal fusion protein studied by Clark et al. (1997). Light gray blocks denote Nod sequences, dark gray blocks denote the KHC component of the Nod-KHC-βgal fusion protein, and black regions denote the βgal or GFP tags. The motor-like domain of Nod falls entirely within the first 320 amino acids at the N-terminus. (B) A drawing of the Nod protein indicating the position of the motor domain, the three HMGN domains, and the HhH(2)/NDD domain. The HMGN and HhH(2)/NDD domains are involved in binding Nod to chromosomes (Afshar et al., 1995b; Cui and Hawley, 2005).

(Matthies et al., 2001). Thus, Nod’s ability to hold chromosomes on the meiotic spindle is unlikely to result from any innate ability to move chromosomes along MT tracks. Although the ability of Nod to hold chromosomes in position might reflect its ability to bridge chromosome arms to the plus ends of nonkinetochores MTs in the spindle and hold them in place, MT tread-milling would presumably draw chromosomes to the poles of the meiotic spindle rather than pushing them toward the metaphase plate.

To better understand how Nod can generate poleward force, it would be helpful to understand how the Nod protein interacts with both chromosomes and MT filaments. In a companion article (Cui and Hawley, 2005), we show that the binding of Nod to the oocyte chromosomes requires the activity of a specific C-terminal motif, referred to as an HhH(2)/NDD domain, which may mediate either nod: DNA or nod:protein interactions. In this article we focus on determining the manner in which Nod interacts with the MTs. Unfortunately, previous studies of the ability of nod, or parts of nod, to localize on microtubule arrays of known polarity within cells have provided confusing results with respect to this question. Clark et al. (1997) demonstrated that a nod-KHC-βgal fusion protein containing the Nod motor-like domain fused to the coiled domain of KHC and β-galactosidase (see Figure 1A) functions as an in vivo reporter for the minus ends of MT arrays. However, using a similar in vivo assay, we show below that full-length Nod-GFP protein, which retains full biological function, localizes in a manner similar to that exhibited by the plus-end-directed motor KHC (Clark et al., 1997; Brendza et al., 2000).

To directly determine whether or not Nod binds to the ends of MTs, and if so, to which end, we have examined the interactions of purified full-length Nod (Nod\(_d1\)) and the Nod motorlike domain (Nod\(_{d1\_d1}\)) with MTs in vitro. We demonstrate that both Nod\(_d1\) and Nod\(_{d1\_d1}\) bind preferentially to the plus ends of the MT and promote MT polymerization. As discussed below, the observation that Nod localizes preferentially to the plus end of MTs suggests a mechanism for its function, similar to the clamped-filament elongation model proposed for actin-based motors (Dickinson and Purich, 2002). This mechanism provides a means for explaining the ability of a protein like Nod, which lacks the capacity for vectorial transport, to propel chromosomes toward the metaphase plate.

MATERIALS AND METHODS

Construction of Nod\(_d1\)-GFP Constructs for Transformation into Drosophila

Full-length wild-type nod cDNA was amplified by PCR and a Smal site was added on the 5’ end of the PCR product. The resulting sequence was cloned into a pEGFP-N1 vector (Clontech, Palo Alto, CA) Smal site by blunt-end ligation to form a Nod\(_d1\)-GFP fusion gene. Subsequently, the Nod\(_d1\)-GFP coding sequence was released by digestion with KpnI and BamHI and ligated into the pUAsp vector (a generous gift from Dr. Fernülle Rørth; Rørth, 1998) double-digested with KpnI and BamHI to form pUAsp-Nod\(_d1\)-GFP. The entire coding sequence was verified on both strands.

Construction of Bacterial Expression Vector Encoding Nod\(_d1\)-GFP

The Nod\(_d1\)-GFP coding sequence was amplified from pUAsp-Nod\(_d1\)-GFP using the primers 5’-ACCGGTCGACAGCGCCGAAAATTAAGGGC-3’ and 5’-AAAGAAAAAAAGCGCGCGCTTGACTGACCGTCCAGGCGG-3’. This PCR product was digested with Sall and NotI and ligated into pET-21a (Novagen, Madison, WI), keeping the coding sequence in frame with the T7 and toHis tags. The T7 tag was subsequently removed by Nhel and Sall double digestion of the vector and replaced with the sequence encoding a Flag tag (DYKDDDDY) using the same sites to form pET-Nod\(_d1\)-GFP.

Construction of Bacterial Expression Vectors Encoding Nod\(_{d1\_d1}\) and Nod\(_{d1\_d1\_d1}\)-GFP

The sequence encoding the N-terminal 318 amino acids of Nod (Nod\(_{d1\_d1}\)) was amplified from wild-type nod cDNA using the primers 5’-ACCGGTCGACAGCGCCGAAAATTAAGGGC-3’ and 5’-AAAGAAAAAAAGCGCGCGCTTGACTGACCGTCCAGGCGG-3’ and double-digested with Sall and NotI. The Nod\(_d1\)-GFP coding sequence in the pET-Nod\(_d1\)-GFP construct was removed by double digestion with Sall and NotI and the digested Nod\(_{d1\_d1}\) PCR product was then cloned into the linearized vector using the Sall and NotI sites. To make a Nod\(_{d1\_d1}\)-GFP bacterial expression vector, the sequence encoding Nod\(_{d1\_d1}\) was amplified from wild-type nod cDNA with the primers: 5’-ACCGGTCGACAGCGCCGAAAATTAAGGGC-3’ and 5’-CAAGCGCGACCTGGCGTGACTGGTGCAGAACCCGC-3’ and double-digested with Sall and BamHI. The pET-Nod\(_d1\)-GFP construct was double-digested with Sall and BamHI to remove the Nod\(_{d1}\) cDNA sequence and the digested Nod\(_{d1\_d1}\) PCR product was cloned into the Sall and BamHI sites. For each of the expression constructs, the entire coding sequence was verified on both strands.

Expression and Purification of Bacterial Expressed Proteins

pET21a vectors carrying constructs encoding either Nod\(_d1\)-GFP, Nod\(_{d1\_d1}\)-GFP, or Nod\(_{d1\_d1\_d1}\) were transformed into BL21(DE3) competent cells (Invitrogen, Carlsbad, CA). A single colony was picked and grown in 2 ml of LB medium plus 100 μg/ml ampicillin at 37°C shaken at 250 rpm overnight. One milliliter of bacterial culture was added to 100 ml of LB medium plus 100 μg/ml ampicillin and grown at 37°C overnight at 250 rpm. Ten milliliters of the 100-ml culture was added to 500 ml LB medium plus 100 μg/ml ampicillin and grown at 37°C to reach OD\(_{600}\) of 0.6. Protein expression was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The bacterial cultures were continuously grown at 20°C for another 4 h. The bacteria were harvested at 6000 rpm for 30 min at 4°C, and the bacterial pellets were stored at −80°C.

The E. coli pellets were resuspended in ice-cold native binding buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, 1% Triton X-100, 10% glycerol, 10 mM ATP, 0.1 mM AEBSF, 1 μg/ml apro tin, 1 μg/ml pepstatin, 1 μg/ml leupeptin) at 5 g/ml. Lysozyme at 1 mg/ml was added to the solution and incubated on ice for 30 min. The solution was passed through a precooled French press twice at 1200−1300 psi. The lysates were treated with DNase I (5 μg/ml) and RNase A (10 μg/ml) on ice for 15–20 min. The lysates were then centrifuged at 18,000 rpm for 30 min at 4°C.
Supernatants were saved and incubated with Ni-NTA agarose beads (Invitrogen) in a 50-ml conical tube with mixing for 2 h at 4°C. The Ni-NTA agarose beads were pelleted at 800 × g for 2 min at 4°C and washed in ice-cold washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 20 mM imidazole, 20 μM β-mercaptoethanol, 1% Triton X-100, 10% glycerol) four times in the 50-ml conical tube. In the last wash, the Ni-NTA agarose beads were loaded onto a 10-ml pull-down column and eluted with elution buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1% glycerol, and 200 mM imidazole). The eluates were dialyzed against ATPase buffer (20 mM HEPES, pH 7.3, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 5% sucrose, 150 mM NaCl, and 50 mM potassium acetate), concentrated using Microcon Centrifugal Filter Devices (Millipore, Bedford, MA), and the protein concentration was measured using a Bradford protein assay (Bio-Rad Laboratories, Richmond, CA).

End Binding Assay
To make polarity marked MTs, both rhodamine-labeled and unlabeled tubulin (Cytoskeleton, Denver, CO) were thawed, adjusted to 1 mM MgGTP, cold-depolymerized, and clarified by centrifugation for 15 min at 14,000 rpm for 4°C. MT seeds were assembled with equal volumes of 20 μM fluorescent rhodamine-labeled and unlabeled tubulin in the presence of 6 μM taxol (pacitaxel, Sigma-Aldrich, St. Louis, MO) at 34°C for 10 min. The highly fluorescent MT seeds were sheathed with a 25-gauge needle (Becton Dickinson, Lincoln Park, NJ) and incubated with 20 μM fluorescent labeled and unlabeled tubulin at the ratio of 1:5 in the presence of 6 μM taxol at 34°C for 10 min. MTs were centrifuged at 14,000 rpm for 15 min and room temperature and resuspended in the same buffer plus 10 mM Pipes, pH 6.9, 5 mM MgCl₂, 1 mM EGTA, 6 μM MgGTP, 1 mM DTT, and 0.1 mM MgGTP.

Nod₁₀₀-GFP or Nod₁₄₆-GFP proteins were allowed to bind to MTs in the presence of 1 mM MgAMPNNP (10 mM Pipes, pH 6.9, 5 mM MgCl₂, 1 mM EGTA, 1.5 mM MgAMPNNP, 1.5 mM magnesium acetate, 1 mg/ml bovine serum albumin, 0.2 mg/ml glucose oxidase, 33 μg/ml catalase, 25 mM glucose, 19.2 μg/ml phospho(en)hexosekinase, 4 mM phospho(en)hexosekinase). The MT-Nod complexes were imaged by fluorescence microscopy and then resuspended using the SoftWoRx package (Applied Precision, Issaquah, WA). The two controls for this experiment are denoted as “no motor” and “GFP.” The no motor control consists of assaying the MT polymerization mixture with no added Nod protein. The GFP control consists of adding recombinant green fluorescent protein (gfpGFP), which was also expressed in E. coli and obtained from BD Biosciences-Clontech, San Jose, CA.

Additional experiments were performed to determine whether there was a preference for Nod binding to the MT plus- or minus-end. Following the protocols of Brum and Brower (2005), the MTs were assembled as described above to obtain polarity marked MTs. The MT-Nod complex (500 mM tubulin, 3 μM taxol, 25 mM Nod₁₀₀-GFP) was preformed in a 10-μl aliquot and immediately fixed in an equal volume of 1% glutaraldehyde in PME (3 μl of the original 10-μl reaction plus 30 μl of 1% glutaraldehyde). An additional 800 μl of PME was added to yield a total volume of 833 μl. An aliquot of 50 μl of the 833-μl reaction was centrifuged through a 10% glycerol cushion onto round 1-mm poly-l-lysine coated glass coverslips. Nod binding was scored as motor binding. For MT end, the MT lattice, or both the MT end and MT lattice, and the binding events on polarity marked MTs were scored for plus- or minus-end binding.

Visual and Sedimentation Analysis of MT Polymerization
For qualitative visual analysis of the polymerization of MTs, both rhodamine-labeled and unlabeled tubulin were thawed, adjusted to 1 mM MgGTP, cold depolymerized, and clarified by centrifugation for 15 min at 14,000 rpm for 4°C. The soluble tubulin was mixed to obtain a final ratio of 1:10 rhodamine-labeled:unlabeled tubulin and adjusted to 20 μM tubulin. Nod proteins at 0 or 50 nM were added with 3 μM of soluble tubulin in the presence of 1 mM MgATP (or MgAMPNNP), 1 mM MgGTP, and 1.5 μM taxol in PMEO (80 mM Pipes, pH 6.9, 5 mM MgCl₂, 1 mM EGTA). The final volume of the reaction was 150 μl. Reactions were initiated by the addition of soluble tubulin to the mix. At the predetermined time points, 8 μl was taken from the tube and centrifused into the observation chamber. Five fields per time point (15-min intervals) were then imaged on an Olympus BX60 epifluorescence microscope (Melville, NY) using a 100× oil immersion objective. Digital images were captured with a Hamamatsu 4742 CCD camera (Bridgewater, NJ) in conjunction with QED In Vivo imaging software (Media Cybernetics, Silver Spring, MD).

For the sedimentation assays to assess MT polymerization, tubulin was treated as described above. Soluble tubulin at 3 μM was incubated with 1.5 μM taxol, 1.5 mM MgATP, 1.5 mM MgGTP, the presence or absence of Nod₁₀₀-GFP or Nod₁₄₆ (0.15 μM of Nod₁₀₀-GFP and 0.3 μM of Nod₁₄₆ at 34°C for 0–10 min, and then the solution was centrifuged in the Airuge (Beckman Coulter, Fullerton, CA) at 20 psi for 30 min. The resulting supernatant and pellet for each reaction were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. The density of the protein bands was measured using Scion Image (Frederick, MD). To verify that the pellets represented MTs, we used rhodamine-labeled and unlabeled tubulin at the ratio of 1:15 and repeated the sedimentation assay. We resuspended the labeled MT pellets in PMEO buffer and evaluated the suspension by fluorescence microscopy.

Statistical Analysis of Polymerization Data
For the sedimentation assays designed to assess MT polymerization, each experiment was run in duplicates, and three independent experiments were performed. The statistical differences between control samples and Nod-treated samples were calculated by Microsoft Excel (Redmond, WA) Student’s t-test. Error bars in Figures 8 and 9 denote the SE of the mean.

Fly Stocks
The pUASp-Nod₁₀₀-GFP construct was introduced into Dro sophila by F element-mediated transformation. Germline expression of Nod₁₀₀-GFP was achieved by expressing the pUASp-Nod₁₀₀-GFP construct under the control of the nanos-Gal4:VPI6 driver (Van Doreen et al., 1998). gkr⁺ and gkr⁻ flies were kindly provided by Dr. Trudi Schüpbach (Princeton University).

Genetic Crosses
We introduced pUASp-Nod₁₀₀-GFP carrying chromosomes into the gkr genetic background to examine Nod₁₀₀-GFP localization. Crosses 1: w¹¹¹⁸; gkr/+; gkr/ gkr/+; gkr/gkr; P{UASp-Nod₁₀₀-GFP}/P{UASp-Nod₁₀₀-GFP} and w¹¹¹⁸; gkr/+; gkr/gkr; P{UASp-Nod₁₀₀-GFP}/P{UASp-Nod₁₀₀-GFP} and w¹¹¹⁸; gkr/+; gkr/gkr; P{UASp-Nod₁₀₀-GFP}/P{UASp-Nod₁₀₀-GFP} flies. Subsequently, w¹¹¹⁸/Y; gkr/+; P{UASp-Nod₁₀₀-GFP}/P{UASp-Nod₁₀₀-GFP} and w¹¹¹⁸/Y; gkr/+; P{UASp-Nod₁₀₀-GFP}/P{UASp-Nod₁₀₀-GFP} flies. Ovaries from these females and gkr/+ controls were dissected and stained with anti-gfp antibody as described in Cui and Hawley (2005).

RESULTS
Functional Nod₁₀₀-GFP Localizes to the Posterior Pole of Stage 9 Oocytes in a Manner That Requires a Properly Organized MT Network
When expressed in nod mutant oocytes under the control of the germline-specific nanos-Gal4:VPI6 driver, the Nod₁₀₀-GFP protein localizes to oocyte chromosomes and fully rescues the defective chromosome segregation phenotype exhibited by nod oocytes (Cui and Hawley, 2005). We show here that Nod₁₀₀-GFP also accumulates at the posterior pole of stage 9 oocytes, as shown in Figure 2. This pattern parallels the localization pattern observed for KHC, a known plus

Figure 2. Nod₁₀₀-GFP localizes to both the oocyte chromosomes and to the posterior pole in stage 9 oocytes. Otherwise wild-type oocytes expressing Nod₁₀₀-GFP were stained with anti-gfp antibody and analyzed by deconvolution microscopy. The open arrowhead indicates Nod₁₀₀-GFP localization to the oocyte chromosomes. The filled arrowhead indicates Nod₁₀₀-GFP localization to the posterior pole of the oocyte. Scale bar, 20 μm.
end-directed motor (Clark et al., 1997; Brendza et al., 2000), but contrasts with that of a Nod-KHC-gal fusion protein, which localizes to the minus ends of MT arrays (Clark et al., 1997). The contradiction between our observations and the localization of Nod-KHC-gal may be resolved by supposing that some component of the Nod-KHC-gal fusion protein other than the Nod motorlike domain, perhaps the KHC sequences, is responsible for minus end-directed localization.

To determine whether the localization of Nod to the posterior pole of stage 9 oocytes requires the integrity of the oocyte MT network, we localized Nod\(_\text{FL}\)-GFP in grk\(_{2B}/\)grk\(_{DC}\) mutant oocytes. Jauschke et al. (2002) demonstrated that the normal organization of MTs in stage 9 oocytes is abolished in grk\(_{2B}/\)grk\(_{DC}\) mutants, as visualized using the MT-associated protein Tau-GFP. Although cytoplasmic Nod\(_\text{FL}\)-GFP is localized at the posterior pole in grk heterozygous stage 9–10A oocytes, this localization was abolished in grk\(_{2B}/\)grk\(_{DC}\) mutant oocytes. Instead, Nod\(_\text{FL}\)-GFP was dispersed throughout the entire egg chamber (Figure 3). These results suggest that Nod\(_\text{FL}\)-GFP accumulation at the posterior pole is dependent on proper MT organization within the oocyte.

However, several lines of evidence suggest that interpreting the significance of this localization in terms of the manner in which Nod interacts with microtubules must be done with some caution. First, the minus-end motor dynein also concentrates at the posterior (Li et al., 1994), albeit in a manner that depends on the function of KHC (Brendza et al., 2002). Second, microtubule imaging shows no evidence of microtubules plus ends being concentrated at the posterior pole. Indeed, although minus ends are highly concentrated at the anterior of the oocyte and near the cortex, the posterior pole is relatively free of microtubules (Cha et al., 2002). Both Cha et al. (2002) and Serbus et al. (2005) have proposed elegant models to explain these discrepancies while still proposing that KHC localizes to the posterior pole because of its capacity to act as a plus end-directed motor and thus push away from the minus ends concentrated at the anterior pole and along the cortex. However, these observations at least raise the possibility that the processes that localize Nod to the posterior pole may not be completely dependent on direct interactions of Nod with the MT. Indeed, Cui and Hawley (2005) have shown that localization of full-length Nod to the posterior pole of the oocyte also requires a functional HhH(2)/NDD DNA-binding domain, raising the possibility that this domain mediates the interaction of Nod with at least one other protein. Thus, in order to directly assess the ability of Nod to bind to the plus ends of MTs we pursued a more direct in vitro approach for studying Nod-MT interactions.

**Figure 3.** Localization of Nod\(_\text{FL}\)-GFP in stage 9 oocytes in which the MT organization is disrupted by mutations in grk. Normal (grk\(+\)) and grk (grk\(_{2B}/\)grk\(_{DC}\)) oocytes expressing Nod\(_\text{FL}\)-GFP were stained with anti-GFP antibody (GFP) and analyzed by deconvolution microscopy. The open arrowhead indicates Nod\(_\text{FL}\)-GFP localization to the oocyte chromosomes. The filled arrowhead indicates Nod\(_\text{FL}\)-GFP localization to the posterior pole of the oocyte. Note that Nod\(_\text{FL}\)-GFP localizes to the oocyte chromosomes in both grk\(+\) and grk oocytes, but only localizes to the posterior pole in grk\(+\) oocytes. Scale bar, 20 μm.

**Figure 4.** Purification of Nod\(_\text{FL}\)-GFP, Nod\(_{318}\) and Nod\(_{318}\)-GFP from *E. coli*. Full-length (Nod\(_\text{FL}\)) or motor domain (Nod\(_{318}\)) constructs were expressed in *E. coli* and purified using Ni-NTA agarose beads. Shown are Coomassie blue-stained SDS-PAGE gels of purified proteins. The (●) denote the full-length proteins, whereas the (●) indicate breakdown products or contaminants. Based on Western blot analysis (unpublished data), the majority of the lower molecular weight bands appear to be the result of breakdown or proteolysis of the Nod-GFP proteins.

**Nod\(_\text{FL}\) and Nod\(_{318}\) Show Preferential Binding to MT Plus Ends In Vitro**

The MT-dependent localization of Nod\(_\text{FL}\)-GFP to the posterior pole suggested that Nod might either be moved to or preferentially bind the plus ends of MTs. To test this possibility, we purified bacterially expressed Nod\(_\text{FL}\)-GFP and Nod\(_{318}\)-GFP proteins, incubated them with polarity marked MTs, and visually assessed the position of Nod binding along the length of the MT. Figure 4 shows the expressed and purified proteins used for these in vitro assays. Although we were successful in purifying Nod\(_{318}\) and Nod\(_{318}\)-GFP to >99% homogeneity, the purification of the full-
length Nod construct (NodFL-GFP) was more problematic. As shown in Figure 4, several additional proteins copurified with NodFL-GFP. Based on Western blot analysis (unpublished data), the majority of the lower molecular weight bands appear to be the result of breakdown or proteolysis of the NodFL-GFP protein.

To determine the ability of these proteins to bind to MTs, we coimmunoprecipitated NodFL-GFP and Nod318-GFP with rhodamine-labeled MTs. A substantial fraction of these MTs were polarity-marked such that the brighter region of fluorescence corresponds to the minus end of the MT while the fainter region corresponds to the region including the plus end (see Figure 5A). We then visualized Nod binding to MTs by measuring the number and position of GFP foci (corresponding to sites of NodFL-GFP and Nod318-GFP binding) along the length of the MTs by fluorescence microscopy. As expected, no foci were observed in the “no motor” control. Furthermore, binding of GFP protein lacking Nod sequences to MTs in this assay was rare, even at high concentrations of protein (57.2 nM), and no instances of end-binding to single MTs were observed. The one example of GFP binding observed involved a GFP focus localized to a site at which the lattices of two MTs appeared to intersect. However, for both NodFL-GFP and Nod318-GFP, Nod-MT complexes were observed and their frequency increased with the concentration of the Nod-GFP protein. Nod318-GFP binds to 2.3–6.5% of MTs at concentrations of Nod318-GFP ranging from 6.1 to 24 nM, whereas NodFL-GFP binds to 3.3–24.4% of MTs at concentrations ranging from 0.19 to 18.9 nM. These observations demonstrate that the Nod-GFP proteins produced in E. coli retain their ability to bind MTs. Moreover, the observation that NodFL-GFP protein binds to more MTs at lower concentrations of protein than does Nod318-GFP suggests that the full-length NodFL-GFP protein has a higher affinity for MTs than does the Nod318 protein, which carries only the motorlike domain of Nod.

We categorized the binding of Nod-GFP proteins to MTs into three classes: end-binding (see Figure 5, A–C), in which the GFP focus defines the end of the MT, lattice binding (see Figure 5, D–F), in which the GFP focus was positioned somewhere along the length of the MT, and junctions (Figure 5, G–I) in which the GFP focus marks a site of intersection between two MTs. In those cases in which the MT was polarity marked, end binding events could be further classified as plus or minus end bindings (see below). Although the images in Figure 5 portray MT-binding events for the NodFL-GFP protein, MT binding data for both NodFL-GFP and Nod318-GFP are summarized in the table embedded in Figure 5. In the case of Nod318-GFP, the majority of MT-binding events involved single MTs at all three concentrations tested, whereas for NodFL-GFP junction events involving two MTs were the most frequently observed class of Nod-MT interaction at all concentrations tested. We will first discuss the cases in which NodFL-GFP or Nod318-GFP interacts with a single MT and then discuss interactions with MT junctions.

Figure 6 displays the MT binding events for both NodFL-GFP and Nod318-GFP as a histogram of Nod-GFP localization along the MTs. The average length of these MTs is 5 μm. We separated the position of the Nod-GFP focus along the microtubule into seven “bins” to denote their position. The first bin, labeled “Tip,” denotes those cases in which the Nod-GFP focus was located at a point between 0 and 7% of the of the length of the MT, and the second bin denotes those cases where the focus fell between 8 and 14% of the length of the MT, and so on. The last bin, labeled “Center,” includes those GFP foci mapping close to or at the center of the MT.

Because we are always measuring the distance from the GFP focus to the nearest end, the position of the focus cannot exceed 50% of the length of the MT. The bin size of seven reflects the fact that for foci denoted as lattice binding events which were close to, but not at, the tip, the distance from focus to tip was at least 7% of MT length. Foci <7% of MT length from the tips were considered end-binding events. Using this binning method to quantitate the position of the GFP focus on the MT, we found that both NodFL-GFP and Nod318-GFP show higher binding affinity to the end of the MT compared with any other location along the MT (see Figure 6). Evidence that the binding of Nod to the MT end occurs preferentially at the plus end is provided in the following section.

In addition to binding at the ends or along the lattice of MTs, NodFL-GFP and Nod318-GFP were often observed to connect two MTs and form structures we denote as junctions (see Figure 5, G–I). The analysis of these junctions further supports our conclusion that Nod binds preferentially to MT ends and suggests that the C-terminal half of Nod also carries an MT-binding domain. The conclusion that the Nod binding in junctions is also often mediated by the binding to MT ends is based on an analysis of the structure of the junctions themselves. Junctions were observed either between two MT ends (with Nod at the junction), between an end and a lattice (as shown in Figure 5, G–I) or between two lattices. The 10 junctions with Nod318-GFP foci may be further classified as 3 end-end junctions, 4 end-lattice junctions, and 3 lattice-lattice junctions. Thus, among the 20 MTs composing these 10 junctions, Nod318-GFP is bound at the end of the MT in 50% of the cases. For NodFL-GFP, a total of 52 MTs were observed as components of 26 junctions. These 26 junction structures may be further classified as 8 end-end junctions, 13 end-lattice junctions, and 5 lattice-lattice junctions, such that of the 52 MTs involved, NodFL-GFP was bound to the end of the MT in 54.7% of the cases.

The argument that Nod possesses a second MT-binding domain within its C-terminus is based on the observation that NodFL-GFP protein clearly possesses a greater ability to bind to or create MT junctions (36–57%) than does the Nod318-GFP protein (8–18%). This greater ability of NodFL-GFP to form or bind to junctions suggests that a second MT-binding domain might exist in the C-terminal half of Nod, such that the NodFL-GFP protein can bind more than one MT fiber. Such a secondary MT binding domain has been identified in the C-terminal (nonmotor) half of the HsKid protein, a human chromokinesin that is similar in structure to Nod (Shiroguchi et al., 2003). Although the ability of Nod to form junctions might also be explained by an ability to form dimers, chromokinesins like Nod and HsKid lack the coiled coil domain that is believed to essential for dimerization (Shiroguchi et al., 2003).

These data allow two general conclusions. First, both NodFL-GFP and Nod318-GFP bind to MTs in vitro, with a strong preference for the ends of the MT. Second, NodFL-GFP has a higher affinity for MT-binding than does Nod318-GFP, apparently as a consequence of a greater ability of NodFL-GFP to interconnect two MTs, a property that may reflect a secondary MT binding site in the C-terminus of Nod. However, even these junction events are also a manifestation of Nod’s preferential ability to bind MT ends, because the majority of junctions involve at least one MT end.

Nod Binds Preferentially to the Plus Ends of MTs

Using those cases in which NodFL-GFP or Nod318-GFP localized to the end of a polarity marked MT, we were able to demonstrate that Nod preferentially binds to the plus ends
Figure 5. NodFL-GFP and Nod318-GFP bind to MTs in vitro. (A, D, and G) Rhodamine-labeled MTs (red). (B, E, and H) NodFL-GFP localization (green). (C, F and I) Merge of the two channels showing NodFL-GFP localization on the MTs. (C) NodFL-GFP localizes to the plus end of a polarity-marked MT. The arrowhead denotes the minus end of the MT. (F) NodFL-GFP localizes to the lattice of a MT. (I) NodFL-GFP localized to the junction between MTs. The table summarizes MT binding scored for NodFL-GFP and Nod318-GFP. Concentrations for NodFL-GFP and Nod318-GFP are listed in the table; the tubulin concentration was 0.5 μM. As can be seen in D–F and G–I, GFP foci were often observed at “bright spots” of rhodamine-tubulin signal along the lengths or at the tips of MTs. Although these associations might indicate a higher concentration of tubulin oligomers at or near the site of Nod binding and the preference for Nod to bind at those sites, we note that “bright spots” are also commonly observed on MTs that do not have bound Nod protein, and thus they are not likely to be a consequence of Nod binding.

Summary of Nod-microtubule binding

<table>
<thead>
<tr>
<th>Nod</th>
<th>Protein</th>
<th>[nM]</th>
<th>Total MT</th>
<th>% MT bound (#)</th>
<th>Lattice</th>
<th>Junction</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>No motor</td>
<td></td>
<td>0.0</td>
<td>335</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>GFP</td>
<td></td>
<td>28.6</td>
<td>535</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>GFP</td>
<td></td>
<td>35.8</td>
<td>423</td>
<td>0.2 (1)</td>
<td>100.0 (1)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>GFP</td>
<td></td>
<td>57.2</td>
<td>763</td>
<td>0.4 (3)</td>
<td>0.0 (0)</td>
<td>100.0 (3)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>NOD318-GFP</td>
<td></td>
<td>6.1</td>
<td>1997</td>
<td>2.3 (45)</td>
<td>42.2 (19)</td>
<td>17.7 (8)</td>
<td>40.0 (18)</td>
</tr>
<tr>
<td>NOD318-GFP</td>
<td></td>
<td>12.1</td>
<td>684</td>
<td>3.6 (24)</td>
<td>33.3 (8)</td>
<td>8.3 (2)</td>
<td>58.3 (14)</td>
</tr>
<tr>
<td>NOD318-GFP</td>
<td></td>
<td>24.2</td>
<td>1179</td>
<td>6.5 (77)</td>
<td>54.5 (42)</td>
<td>10.3 (10)</td>
<td>32.4 (25)</td>
</tr>
<tr>
<td>NOD6T-GFP</td>
<td></td>
<td>0.2</td>
<td>213</td>
<td>3.3 (7)</td>
<td>14.2 (1)</td>
<td>57.1 (4)</td>
<td>28.4 (2)</td>
</tr>
<tr>
<td>NOD6T-GFP</td>
<td></td>
<td>1.0</td>
<td>427</td>
<td>6.3 (27)</td>
<td>22.2 (6)</td>
<td>44.4 (12)</td>
<td>33.3 (9)</td>
</tr>
<tr>
<td>NOD6T-GFP</td>
<td></td>
<td>5.7</td>
<td>284</td>
<td>16.5 (47)</td>
<td>25.5 (12)</td>
<td>51.1 (24)</td>
<td>23.4 (11)</td>
</tr>
<tr>
<td>NOD6T-GFP</td>
<td></td>
<td>18.9</td>
<td>135</td>
<td>24.4 (33)</td>
<td>27.2 (9)</td>
<td>36.4 (12)</td>
<td>36.4 (12)</td>
</tr>
</tbody>
</table>

* There are end-end, end-lattice and lattice-lattice junctions. All the MTs are counted in the junctions.
which Nod\textsubscript{318}-GFP bound to the MT end (71.8\%) was approximately twofold higher than observed in the experiment presented in Figure 5, in which Nod\textsubscript{318}-GFP was mixed with MTs only in the presence of AMPPNP. Moreover the ratio of plus-end (52) to minus end (3) binding events was \(\sim 17:1\). The difference in these two sets of results likely reflects the fact that by mixing Nod\textsubscript{318}-GFP and MTs in the presence of AMPPNP we are failing to trap the Nod complex at the site of the initial binding, and AMPPNP may be allowing Nod to migrate along the length of the MT. However, by fixing with glutaraldehyde immediately after mixing, as we do in Figure 7, we are capturing the initial sites of Nod MT interactions. Taken together, both experiments argue strongly that when binding to the MT end, Nod has a strong preference for the plus end.

**Nod\textsubscript{FL-GFP} and Nod\textsubscript{318} Promote MT Polymerization In Vitro**

The preferential binding of Nod to ends, and specifically the plus ends, of MTs suggested that Nod might play a role in controlling MT polymerization. To address this possibility, we set out to test the ability of Nod\textsubscript{FL-GFP} and Nod\textsubscript{318} to facilitate this process. To test the ability of the Nod motorlike domain to mediate MT polymerization, we incubated Nod\textsubscript{318} with soluble rhodamine-labeled tubulin in the presence of MgATP or MgAMPPNP. At times 0 and 30 min, we visualized the presence or absence of polymerization by microscopic examination. As shown in Figure 8, there are many more MTs formed by 30 min in Nod-treated samples than are formed in the no motor control. The observation that Nod-promoted MT assembly occurred in the presence of either MgATP or MgAMPPNP suggests that Nod\textsubscript{318} does not require ATP turnover to promote MT polymerization. To control for the possibility that some of the observed MT polymerization might be due to the presence of bacterial proteins, we repeated this experiment by performing the MT polymerization experiment using extracts from *E. coli* cultures that did or did not express Nod\textsubscript{318}. Although a high degree of MT polymerization was observed in the presence of Nod\textsubscript{319}, little or no polymerization was observed in the presence of *E. coli* extract alone (see Supplementary Figure 1).

We used sedimentation analysis to quantify the ability of the Nod motorlike domain (Nod\textsubscript{318}) and full-length Nod of MTs. As shown in Figure 5, we found that Nod bound twice as frequently to the plus versus the minus end of the MT for both Nod\textsubscript{FL-GFP} and Nod\textsubscript{318-GFP}. The ratio of plus end/minus end binding events for Nod\textsubscript{FL-GFP} was 15/8, and for Nod\textsubscript{318-GFP} the ratio was 8/4. However, because the number of instances in which Nod was bound to the end of a polarity-marked MT was small, we repeated these experiments using a different method (glutaraldehyde fixation) for trapping the collision complex between Nod\textsubscript{318-GFP} and polarity marked MTs. The results of this experiment are presented in Figure 7. In this experiment the frequency with

![Figure 6. Distribution of Nod binding sites along MTs. The MT was divided into 7 segments from either end, such that 0 indicates either MT tip and 50, the center of the MT. Thus the first “bin” denotes those cases where the Nod focus fell at a point between 0 and 7% of the length of the microtubule, the second bin denotes those cases where the focus fell between 8 and 14% of the length of the microtubule, and so on. Because we are always measuring the distance from the GFP focus to the nearest tip of the MT the distance cannot exceed 50%. The bin size of seven reflects the fact that for those foci which were close to, but not at, the tip, the minimum distance from focus to tip was 7% of the length. The frequency of Nod\textsubscript{FL-GFP} and Nod\textsubscript{318-GFP} localization to each segment is plotted. The MTs scored for this localization are listed in the table in Figure 5.](image)

![Figure 7. Trapping of the Nod\textsubscript{318-GFP} collision complex by glutaraldehyde fixation. (A–C) A polarity marked MT denoted by a bright seed at the minus end (arrowhead in A) bound at the dimmer plus-end by Nod\textsubscript{318-GFP}. The average length of the MTs counted was 4.63 \(\pm\) 0.18 (SE). The tubulin concentration was 0.5 \(\mu\)M.](image)
(NodFL-GFP) to mediate MT polymerization. Soluble tubulin was incubated with or without NodFL-GFP or Nod318, centrifuged, and subjected to SDS-PAGE to determine the fraction of tubulin that remained in the supernatant in comparison to the fraction that sedimented in the pellet. As shown in Figure 9A, both NodFL-GFP and Nod318 result in a statistically significant increase (p < 0.01) in tubulin partitioning to the pellet when compared with the no motor controls. Similar results were also obtained using a Nod318* GFP construct (unpublished data). To confirm that the partitioning of tubulin to the pellets represents Nod-promoted MT polymerization, we repeated the sedimentation assay.

Figure 8. Nod promotes MT polymerization in vitro. Soluble labeled tubulin, 3 μM, was complexed with 50 nM Nod318 in the presence of MgATP and the reaction was incubated for varying time points, at which time, 8 μl was extracted and MT polymerization was visually assayed on a fluorescence microscope. (A, C, and E) time 0; (B, D, and F) 30 min. (B) Little MT polymerization is observed in the absence of any motor; (D) In the presence of Nod318 and MgATP, polymerization of individual MTs can be observed; (F) In the presence of Nod318 and MgAMPPNP, polymerization is also seen with bundling of the MTs.
with fluorescently labeled tubulin and resuspended the pellets for direct microscopic examination. As shown for Nod\textsubscript{FL}-GFP in Figure 9C, the resuspended pellets are comprised of large numbers of MTs rather than aggregates of soluble tubulin.

If Nod-promoted MT assembly is functionally significant, we would expect there to be a time-dependence to the process. Figure 10 shows Nod\textsubscript{318}-promoted MT assembly using the sedimentation assay. The results reveal a significant increase (p < 0.05) in the fraction of tubulin that partitions to the pellet over time, whereas no increase in the fraction of tubulin sedimentation was seen in the absence of Nod. Therefore, Nod exerts its ability to stimulate MT polymerization in a time-dependent manner.

DISCUSSION

The data presented above reveal two critical insights into Nod function: namely that full-length Nod localizes to the ends of MTs in vitro and that Nod promotes MT polymerization. Our conclusion that Nod binds preferentially to plus ends in vivo is supported by three observations: 1) Nod localizes to the posterior pole of stage 9 oocytes, in a manner similar to the plus-end-directed motor KHC; 2) the preferential binding of Nod to MT plus ends in vitro; and 3) Nod-promoted MT polymerization in vitro. Given that the MTs in the oocyte spindle are arranged with their plus ends at or near the metaphase plate (Riparbelli and Calliani, 2005), these data suggest a straightforward mechanism by which Nod, when bound to the arms of chromosomes, can generate the force required to push chromosomes arms toward the metaphase plate (Matthies et al., 1999; Goshima and Vale, 2003). Indeed, we propose that Nod proteins bound along the arms of the chromosome cause the extension of MT plus ends by polymerization and that it is the growth in the MTs that serves to literally “push” those arms toward the metaphase plates. If correct, this mechanism provides at least one biochemical explanation for the “polar ejection force” (Rieder et al., 1986).

In the experiments presented, Nod promotes polymerization of MTs in the presence of both MgATP and MgAMP-PNP, suggesting that ATP turnover is not required for the addition of tubulin subunits. This observation is perhaps not surprising given the observation by Matthies et al. (2001) that the affinity of Nod-ATP for MTs is similar to, and indeed slightly less than, the affinity of Nod-ADP for MTs. (Compare this to conventional kinesins in which the affinity of the motor-ATP complex for MTs is ~40–50 times greater than the affinity of the motor-ADP complex for MTs.) Nod ATPase activity may not be critical for the MT polymerization activity but it is essential for the ability of Nod to faithfully segregate chromosomes (Rasooly et al., 1991, 1994; Matthies et al., 2001). This apparent contradiction can be reconciled by the following model.

1) Nod MT polymerization activity also functions to stabilize the plus ends of growing MTs. Indeed, plus end binding proteins have this property (Howard and Hyman, 2003). In vivo, MTs are highly dynamic structures undergoing fluctuations between growth and rapid shortening; these dynamics are highly regulated by MT-binding proteins, which bind to the MT ends to facilitate stabilization or destabilization of the MT (Desai and Mitchison, 1997; Desai et al., 1999, Howard and Hyman, 2003). Nod may act as a stabilizing protein by binding to the chromosomes with its C-terminus and to the MT plus end with its N-terminal kinesin motor domain.

Figure 9. Sedimentation analysis of the effect of Nod on MT polymerization. (A) Both Nod\textsubscript{318} (300 nM) and Nod\textsubscript{15} (150 nM) incubated with 3 \(\mu\)M soluble tubulin promote MT polymerization over time in the presence of MgATP. The reactions were centrifuged, and the supernatant and pellet at equal volume for each reaction were analyzed by SDS-PAGE. The amount of tubulin that partitioned to the supernatant and to the pellet were quantified (see gel slices). Error bars, SE of the mean. (B and C) To ensure that the tubulin that partitioned to the pellet was polymerized tubulin (MTs) and not aggregates of soluble tubulin, the experiment was repeated using rhodamine-tubulin, and the pellets were resuspended and examined using fluorescence microscopy. Each experiment was performed in duplicate on three separate occasions. The value presented in the histogram (A) is the average of those six repetitions. Error bars, SEM. The statistical differences between control samples and Nod-treated samples were calculated by Microsoft Excel Student’s t-test.

Figure 10. Nod promotes MT polymerization in a time-dependent manner. Soluble tubulin, 3 \(\mu\)M, and 300 nM Nod\textsubscript{318} incubated with MgATP polymerizes tubulin as a function of time as indicated by the increase of tubulin partitioning to the pellet (see gel slices). Each experiment was performed in duplicate on three separate occasions. The value presented in the histogram is the average of those six repetitions. Error bars, SEM. The statistical differences between control samples and Nod-treated samples were calculated by Microsoft Excel Student’s t-test.
2) By stabilizing the plus ends, Nod allows new tubulin dimers to be added to these ends. The addition of a new dimer leads to GTP hydrolysis in the MT polymer and a new GTP cap. This GTP cap may then be the new binding site for Nod.

3) ATP turnover by Nod could regulate in part the dynamics at the MT plus end by allowing Nod to detach from the elongated MT at the appropriate time and allowing rebinding to the plus end. This mechanism of binding the MT plus end, stabilizing the plus ends, then allowing subunit addition, would account for the in vivo observations that suggest Nod acts to “push” chromosomes away from the poles during meiotic spindle formation.

The various aspects of this model help to explain how Nod, a chromokinesin-like protein that lacks the capacity for vectorial transport, can nonetheless provide the force that maintains achiasmate chromosomes near the metaphase plate during spindle elongation at prometaphase I of meiosis.

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