Physiological Evidence for Involvement of a Kinesin-related Protein during Anaphase Spindle Elongation in Diatom Central Spindles

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Abstract. We have developed a new model system for studying spindle elongation in vitro using the pennate, marine diatom *Cylindrotheca fusiformis. C. fusiformis* can be grown in bulk to high densities while in log phase growth and synchronized by a simple light/dark regime. Isolated spindles can be attained in quantities sufficient for biochemical analysis and spindle tubulin is ~5% of the total protein present. The spindle isolation procedure results in a 10-fold enrichment of diatom tubulin and a calculated 40-fold increase in spindle protein. Isolated spindles or spindles in permeabilized cells can elongate in vitro by the same mechanism and with the same pharmacological sensitivities as described for other anaphase B models (Cande and McDonald, 1986; Masuda et al., 1990). Using this model, in vitro spindle elongation rate profiles were developed for a battery of nucleotide triphosphates and ATP analogs. The relative rates of spindle elongation produced by various nucleotide triphosphates parallel relative rates seen for kinesin-based motility in microtubule gliding assays. Likewise ATP analogs that allow discrimination between myosin-, dynein-, and kinesin-mediated motility produce relative spindle elongation rates characteristic of kinesin motility. Also, isolated spindle fractions are enriched for a kinesin related protein as identified by a peptide antibody against a conserved region of the kinesin superfamily. These data suggest that kinesin-like motility contributes to spindle elongation during anaphase B of mitosis.

A central goal of mitosis research is to identify the molecular motors responsible for chromosome segregation. This problem is exacerbated by the observation that multiple motility events occur in association with spindle microtubules (MTs)[1] during the course of mitosis. Functional models where various mitotic movements could be isolated and analyzed biochemically would greatly aid in the identification of the mechanochemical enzymes that orchestrate chromosome movements (Cande, 1989).

The pharmacology of chromosome-to-pole movement (anaphase A) and spindle elongation (anaphase B) are distinctly different (Cande, 1982; Lee, 1989), suggesting that these processes use different motors. Even with respect to spindle elongation two classes of mechanisms appear to contribute to pole from pole movements: (*a*) pushing forces due to a mechanochemical system in the spindle midzone (Cande, 1989); (*b*) pulling forces mediated through the astral microtubule arrays that exert force on the spindle (Aist and Berns, 1981). While pulling forces may be restricted to organisms that have extensive astral microtubule arrays or whose nuclei undergo nuclear migration, internally generated spindle forces may be universal to all eukaryotic cells and play a role not only during anaphase but also during spindle formation (Hogan and Cande, 1990).

Isolated spindles from diatoms (Cande and McDonald, 1985) and sea urchins (Rebhun and Palazzo, 1988; Palazzo et al., 1991), and permeabilized cells from mammals (Cande, 1982) and fission yeast (Masuda et al., 1990) have been successfully used as functional models for anaphase B and have demonstrated that forces generated from within the spindle are sufficient for spindle elongation in vitro. In diatom and yeast cell models spindles elongate in vitro due to mechanochemical forces generated in the zone of microtubule overlap that mediate the sliding apart of one set of antiparallel MTs against the other (Cande and McDonald, 1985; Masuda et al., 1990). Microtubule rearrangements consistent with a sliding mechanism have been shown in vivo for a variety of organisms including diatoms, yeast, *Dictyostelium*, mammalian cells and protists (reviewed in McDonald, 1989). The interactions between antiparallel polar MTs in the zone of microtubule overlap that drive spindle poles apart could involve one to several MT plus end-directed motors.

Although genetic systems have recently provided good evidence that link novel kinesin-like motors to mitotic spindle movements (Enos and Morris, 1990; Hagan and Yanagida, 1990; Meluh and Rose, 1990; Endow et al., 1990; Zhang et

1. *Abbreviations used in this paper:* BtTb, biotinylated tubulin; IS, isolated spindle extract; MT, microtubule; PC, permeabilized cell extract; NTP, nucleotide triphosphate; RBC, ribulose-1,5-bisphosphate carboxylase; WC, whole cell extract.

al., 1990), mutant phenotypes have been difficult to analyze biochemically and cytologically. Biochemical evidence is required to confirm these predictions and to sort out specific motor functions. One approach is to use in vitro models that are amenable to biochemical dissection. Existing cell models for studying anaphase A or anaphase B have not been useful, either because of the complexity of the model or the inability to obtain spindles in large quantities. To overcome these problems we have developed a new model system for studying spindle elongation in vitro using the pennate, marine diatom *Cylindrotheca fusiformis*. *C. fusiformis* provides both a highly sensitive and reproducible system for analysis of in vitro spindle elongation as well as spindle quantities sufficient for biochemical analysis. Permeabilized cell models were used to study relative spindle elongation rates supported by a battery of nucleotide triphosphates (NTPs) and ATP analogs. We have compared the rates obtained to published rates for dynein and kinesin motility that are supported by the same ATP analogs (Shimizu et al., 1991). The spindle isolation procedure was monitored on immunoblots with an antibody against tubulin as well as a peptide antibody made against a region in the motor domain that is conserved among the kinesin superfamily (Sawin et al., 1992). The isolated spindle fraction is enriched in tubulin as well as a polypeptide that cross reacts with the peptide antibody.

Materials and Methods

Diatom Growth and Synchrony

Details of cell culture and results of drugging will be reported elsewhere (Hogan et al., 1993, in press). Briefly, cultures of *C. fusiformis* were kindly provided by Dr. Ben E. Volcani (Scripps Institute for Oceanography, La Jolla, CA). Diatom cultures were maintained in natural sea water supplemented with F/2 medium (Guillard, 1975) and grown at 22°C. A synchronized population of cells was obtained by seeding 10, 8 liter carboys of sterilized sea water supplemented with F/2 and growing them in continuous light for 48 h with bubbled 3% CO_2 and vigorous stirring. Then lights were turned off for 24 h. 6 h after the initiation of a second light period cells synchronously divided over the following 5 h (Darley and Volcani, 1971). To collect cells with metaphase spindles cultures were made 10^{-7} M nocodazole. At the beginning of nocodazole treatment an extensive interphase MT array emanated from the cell center (Fig. 1 *a*). However, after 3 h in 10^{-7} M nocodazole interphase arrays had disappeared (Fig. 1 *b*, top cell) while metaphase central spindles formed that were resistant to depolymerization (Fig. 1 *b*). Cells were harvested 3–4 h after drug treatment. 30–50% of the cells contained spindles at the time of harvest. The spindles averaged 4–5 μm in length (Fig. 1 *b*) and EM suggests that half-spindles contain at least 10 MTs and that the zone of MT overlap is about 0.5 μm (not shown).

Permeabilized Cell Models

After allowing spindles to accumulate in synchronized populations, cells from ~80 liters of culture were harvested and concentrated to 1 liter in ~45 min using a Pellicon tangential flow filtration system with 0.45-μm Durapore filters (Millipore Corp., Bedford, MA). Cells were then pelleted at 2,500 *g* in a tabletop centrifuge (Sorvall RT6000B; DuPont Company, Wilmington, DE) and washed two times in 1 liter of an isotonic buffer (Cande and McDonald, 1986) that included 10 mM EGTA. Cells were permeabilized by gentle shaking on ice for 15 min in 500 ml PMEG (50 mM Pipes, pH 7.0, 5 mM $MgSO_4$, 5 mM EGTA, 40 mM β-glycerophosphate, 100 μM TROLOX, 1 mM DTT, a proteinase inhibitor cocktail [Masuda et al., 1988], and 1 mM PMSF) with 1% Triton X-100 and 3% DMSO added. For some experiments 20 μM ATPγS (Boehringer Mannheim Corp., Indianapolis, IN) was included in the buffer at this point. Permeabilized cells were pelleted as before and the same treatment was repeated two more times until most of the chlorophyll was depleted from the cells. The permeabilized cells were then washed twice in 500 ml PMEG + 3% DMSO, resuspended in 50 ml PMEG + 3% DMSO, and either used immediately or aliquoted and frozen in liquid N_2 for later use. Spindles can be stored in liquid N_2 for at least 1 yr without any loss of functional capability.

Spindle Isolation

To isolate spindles, 80 liters of synchronized, nocodazole-treated cells were concentrated and washed as above. The pellet of cells (20–25 g) was resuspended in 260 ml PMEG containing 3% DMSO and mixed with 1/3 vol 0.1-mm glass beads in a 350-ml Bead Beater chamber. Cells were homogenized by bead beating (Bead Beater; Biospec Prod., Bartlesville, OK) on ice in three, 30-s bursts with 30 sec rests in between. The homogenate was filtered through 30 μm Nitex (Tetko Inc., Elmsford, NY) to remove glass beads and debris and then the filtrate was centrifuged at 200 *g* for 10 min at 4°C to remove diatom frustules and remaining glass beads. The supernatant was decanted and centrifuged at 2,600 *g* in a HB-4 rotor (Sorval, DuPont, Wilmington, DE) for 40 min at 4°C to remove chloroplasts and nuclei. The supernatant was collected, made 1% Triton X-100, and stirred for 20 min at 4°C. At this point the detergent-treated spindle supernatant could be processed further or frozen in liquid N_2 for at least 1 y. Spindles were washed free of soluble proteins with 600 ml PMEG containing 1% Triton X-100 and 3% DMSO followed by 250 ml PMEG containing 3% DMSO using a Minitan tangential flow filtration unit equipped with 0.2-μm Durapore filters (Millipore Corp., Bedford, MA). The resulting retentate that contained spindles was underlayered with 20% sucrose (in PMEG) and centrifuged at 8000 *g* for 10 min at 4°C in a HB-4 rotor to collect spindles at the buffer/sucrose interface. This final spindle fraction could then be spun onto coverslips for in vitro assays or used for biochemical analysis.

Physiological and Pharmacological Experiments

For assessment of nucleotide specificity of spindle reactivation, and for the characterization of inhibitors of spindle elongation, a 0.5-ml aliquot of permeabilized cells was thawed from liquid N_2 and pelleted through 10 ml PMEG containing 3% DMSO. DMSO was washed from cells by resuspending and pelleting them in 10 ml PMEG, followed by resuspension in 2 ml PMEG. 10–30 μl of this suspension was spun onto poly-L-lysine coated coverslips through 3 ml PMEG. Coverslips were then drained of excess buffer, incubated with 40 μl of the appropriate solution(s) for the times indicated, then fixed. Alternatively, 20–50 μl isolated spindles from the buffer/sucrose interface were spun onto acid cleaned coverslips and subsequently treated as just described. A range of nucleotide and nucleotide analogs were screened for their ability to support spindle reactivation. The synthesis and purification of these compounds as well as the abbreviations used in Fig. 4 *b* have been described previously (Shimizu et al., 1991). The MT-based motility inhibitors used in this study were: (*a*) sodium orthovanadate (Accurate Chem. and Sci. Corp., Hicksville, NY) prepared as a 10 mM stock in 50 mM Hepes, pH 7.5, and boiled before use; (*b*) *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) prepared as a 100 mM stock in dH_2O; and (*c*), 5′-adenylyl-imidodiphosphate (AMP-PNP) (Boeringer-Mannheim Corp., Indianapolis, IN) prepared as a 100 mM stock in dH_2O. For experiments in which exogenous biotinylated tubulin (BtTb) was incorporated into spindles, permeabilized cells on coverslips were stored on ice in 30% glycerol made in PMEG for 10 min or less. Just before use coverslips were washed free of glycerol with PMEG and excess buffer removed by blotting coverslips with a Kimwipe. Coverslips were then incubated for 5 min at room temperature in PMEG containing 0.2 mM GTP and 20 μM bovine brain tubulin that had been biotinylated (Hyman et al., 1990). On some coverslips 1 mM ATP was included in this buffer. In other cases the solution was made 1 mM ATP after the 5-min incubation. Controls were not exposed to ATP.

Immunostaining and Microscopy

Coverslips were fixed 10 min in 0.1% glutaraldehyde, 0.05% paraformaldehyde in PME, pH 7, and then rinsed in PBS, reduced 5 min in 1 mg/ml $NaBH_4$ in 50% methanol, and finally rehydrated in PBS. BtTb was visualized with fluorescein-avidin (Vector Laboratories, Inc., Burlingame, CA). Total tubulin was visualized with either a monoclonal anti-sea urchin α-tubulin (a gift from Dr. David Asai, Purdue University) or rabbit anti-soy bean tubulin (a gift from Dr. Richard Cyr, Penn State University, University Park, PA) and appropriate FITC- or rhodamine-conjugated secondary antibodies (Sigma Chem. Co.). Thiophosphorylated epitopes were visualized using a mAb (Wordeman and Cande, 1987) and FITC-conjugated anti-mouse IgG (Sigma Chemical Co.). Before mounting, coverslips were dipped

briefly in PBS containing 1 μM 4',6-diamidino-2-phenyl-indole (DAPI) (Sigma Chemical Co.). Coverslips were mounted on slides in 90% glycerol containing 1 mg/ml 1,4-Diazabicyclo[2.2.2]-octane (Aldrich Chem. Co., Inc., Milwaukee, WI) in PBS, pH 8.0, and sealed. Slides were observed and photographed on a Zeiss Photoscope using either a Zeiss 40× plan-Neofluar objective (NA 0.9), or a Zeiss 100× Neofluar objective (NA 1.30) (Carl Zeiss, Inc., Thornwood, NY) with epifluorescence illumination and appropriate filters for FITC, rhodamine, or DAPI detection. Images were recorded on T-Max 400 film (Eastman Kodak Co., Rochester, NY) that was developed in D-19 developer (Eastman Kodak Co.).

Extracts and Tubulin Measurement

For determination of polypeptides present in the various spindle preparations, cells from one-half of a synchronized 80 liter preparation were bead beaten, filtered, and spun 10 min at 200 *g* as described above (see spindle isolation). Part of the resulting supernatant was saved and constituted the whole cell extract (WC). The remaining supernatant was processed through tangential flow filtration (see spindle isolation) and then spindles were pelleted at 10,000 *g* 20 min at 4°C. Spindles were resuspended in a small volume of PMEG containing 0.5 M NaCl and sonicated on ice. After sonication the solution was cleared by spinning at 16,000 *g* 15 min at 4°C and finally dialyzed against several changes of PMEG. The resulting solution was saved as isolated spindle extract (IS). The remaining cells from the preparation were processed as described above (see permeabilized cell models). Permeabilized cells were pelleted, resuspended in a small volume of PMEG and the suspension made 0.5 M NaCl. The suspension was then sonicated on ice and centrifuged at 150,000 *g* 30 min at 4°C. The resulting supernatant was dialyzed against several changes of PMEG and saved as permeabilized cell extract (PC). For each extract total protein was measured (Bradford, 1976) and tubulin was quantified by ELISA as follows. A dilution series was made of WC, PC, and IS extracts in TEN (50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl) and air dried overnight in wells of a microtiter plate (ELISA wells; Corning Glass Works, Corning, NY). DEAE-purified bovine brain tubulin diluted in TEN was also dried to wells to yield a standard curve over a range of 25–800 ng tubulin/well. Wells were blocked with 2% normal sheep serum in TEN for 1 h and then incubated with anti-tubulin (1:1,000 in blocking buffer) for 1 h at 35°C. Wells were washed three times with TEN and then incubated 1 h at 35°C with peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:1,000 in blocking solution. After washing three times with TEN, wells were developed with 0.08% *o*-phenylenediamine and 0.5% H_2O_2 in 50 mM citric acid, 0.1 M Na_2HPO_4, and absorbances were read at 450 nm.

PAGE and Immunoblotting

WC, PC, and IS extracts were subjected to 4–10% SDS-PAGE as described (Hogan, 1987). For immunoblotting polypeptides were transferred to nitrocellulose by semi-dry electroblotting for 1.5 h at 0.8 mA cm^{-2} nitrocellulose. Blots were processed as described previously (Hogan, 1987). Primary antibodies used as probes consisted of monoclonal mouse anti-tubulin (Cande and McDonald, 1985), affinity purified rabbit anti-ribulose-1,5-bisphosphate carboxylase (Plumley et al., 1986), and affinity purified rabbit anti-LAGSE (Sawin et al., 1992).

Results

C. fusiformis Central Spindle Organization and Mechanism of Elongation Is Similar to That Found in Other Spindles

Metaphase central spindles resembled functional spindle models from the diatom *Stephanopyxis turris* both morphologically and with respect to location of phosphorylated proteins. Cells permeabilized in the presence of ATPγS showed thiophosphate incorporation into components located in the spindle midzone and at the poles (Fig. 1 *c*). Identical sites of thiophosphate incorporation were seen previously in isolated spindles from *S. turris* (Wordeman and Cande, 1987; Wordeman et al., 1989). These observations show that spindle organization is shared between the two diatom species and suggest that both spindles may contain similar central spindle components.

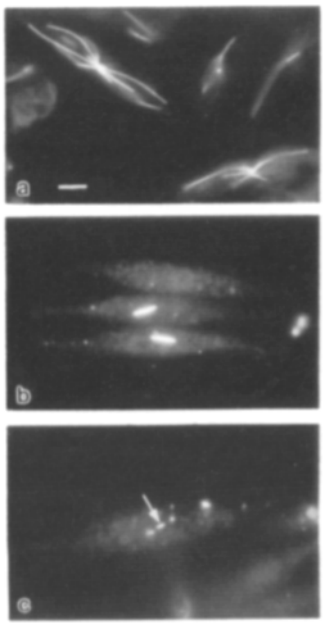

Figure 1. (*a* and *b*) Synchronously dividing cells were detergent-permeabilized and stained with anti-tubulin (*a*) at the beginning of and (*b*) 3 h into nocodazole treatment. Most cells contained an interphase array of MTs before drug treatment (*a*). A low level of nocodazole (10^{-7} M) caused interphase arrays to depolymerize (*b*, top cell) and allowed metaphase spindles to accumulate in 30–50% of the population (*b*, bottom two cells). (*c*) Thiophosphate incorporation into spindles. Cells were permeabilized in 20–50 μM ATPγS. Thiophosphorylated epitopes, visualized by a mAb, were located primarily at the zone of MT overlap (*arrow*) and also at the poles. Bar, 5 μm.

To determine whether spindles in permeabilized cells retained the components required for anaphase B, we assayed the preparation for spindle elongation in vitro. After two washes to remove DMSO, cells were treated with 1 mM ATP for 5 min at room temperature, fixed, and changes in spindle morphology were visualized by indirect immunofluorescence using anti-tubulin. Over the course of several hundred experiments 75–90% (typically ~85%) of the spindles exhibited a gap, or a bend and a gap between the two half-spindles after ATP treatment (Fig. 2, A, *b* and *c*, and *B*). As described previously (Cande and McDonald, 1985, 1986) this rearrangement of MTs in diatom central spindles can result from spindle elongation in vitro. However, one concern was that the gap was generated by half spindle MTs depolymerizing poleward from their free plus ends after ATP addition, rather than by sliding apart of the two half spindles. To address this question exogenously added BtTb was allowed to polymerize onto free plus ends of MTs in the absence of ATP. This treatment labeled diatom MT ends at either edge of the zone of MT overlap with a tubulin polymer that could be distinguished from diatom tubulin. In addition, exogenous tubulin polymerized into new astral arrays at the spindle poles. Figs. 2 A, *d–f*, and *B* show the positions of the plus MT ends in each half-spindle (marked by the incorporated BtTb) before and after ATP treatment. When biotin was visualized before ATP addition the poles as well as a band on either side of the MT overlap zone were evident (Figs. 2 A, *d*, and *B*). Subsequent to ATP addition many spindles were bent or broken, and the two bands of biotin seen in unreactivated spindles merged into one band in the spindle midzone (Figs. 2 A, *e* and *f*, and *B*). In the bent and broken spindles, the plus ends of MTs retained their BtTb caps. This indicated that the bent and broken spindles resulted from MTs sliding apart rather than from MT depolymerization. All of the ATP-induced changes in spindle morphology observed were consistent with MT rearrangements that resulted from sliding apart of the two half-

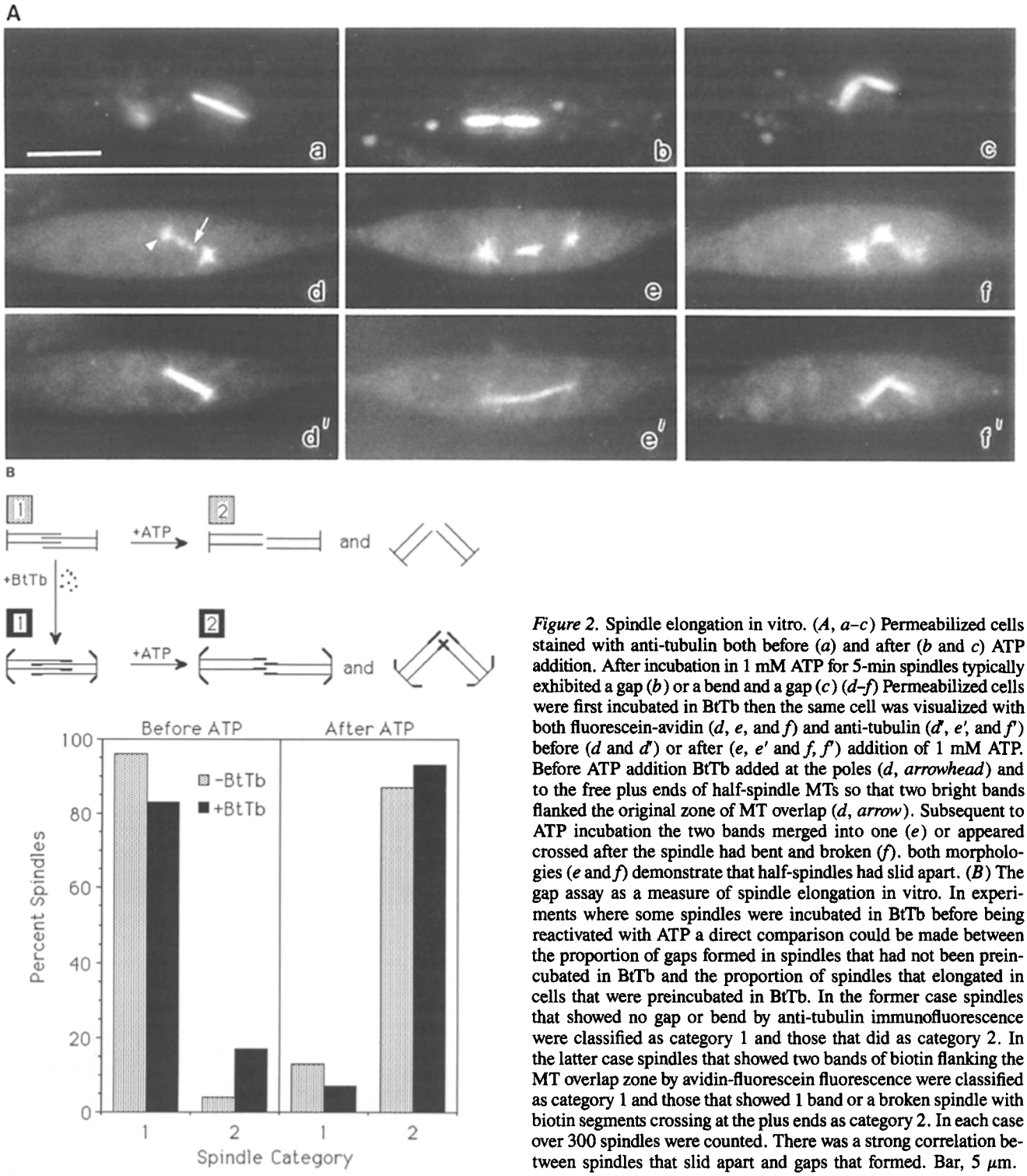

Figure 2. Spindle elongation in vitro. (*A*, *a–c*) Permeabilized cells stained with anti-tubulin both before (*a*) and after (*b* and *c*) ATP addition. After incubation in 1 mM ATP for 5-min spindles typically exhibited a gap (*b*) or a bend and a gap (*c*) (*d–f*) Permeabilized cells were first incubated in BtTb then the same cell was visualized with both fluorescein-avidin (*d*, *e*, and *f*) and anti-tubulin (*d'*, *e'*, and *f'*) before (*d* and *d'*) or after (*e*, *e'* and *f*, *f'*) addition of 1 mM ATP. Before ATP addition BtTb added at the poles (*d*, *arrowhead*) and to the free plus ends of half-spindle MTs so that two bright bands flanked the original zone of MT overlap (*d*, *arrow*). Subsequent to ATP incubation the two bands merged into one (*e*) or appeared crossed after the spindle had bent and broken (*f*). both morphologies (*e* and *f*) demonstrate that half-spindles had slid apart. (*B*) The gap assay as a measure of spindle elongation in vitro. In experiments where some spindles were incubated in BtTb before being reactivated with ATP a direct comparison could be made between the proportion of gaps formed in spindles that had not been preincubated in BtTb and the proportion of spindles that elongated in cells that were preincubated in BtTb. In the former case spindles that showed no gap or bend by anti-tubulin immunofluorescence were classified as category 1 and those that did as category 2. In the latter case spindles that showed two bands of biotin flanking the MT overlap zone by avidin-fluorescein fluorescence were classified as category 1 and those that showed 1 band or a broken spindle with biotin segments crossing at the plus ends as category 2. In each case over 300 spindles were counted. There was a strong correlation between spindles that slid apart and gaps that formed. Bar, 5 μm.

spindles. There was also a strong correlation between the proportion of spindles with ATP-induced gaps in experiments where BtTb was not used to delineate MT ends and the proportion of spindles with BtTb plus ends that slid apart after ATP addition (Fig. 2 *B*). These results validated the gap assay as a measure of spindle elongation in vitro. The gap assay was used subsequently to assess the response of the spindle motor to other NTPs and ATP analogs as well as to inhibitors of MT-based motility.

Isolated Spindle Preparation and ATP-dependent Elongation of Isolated Spindles

Spindle fractions isolated from populations of diatoms in metaphase were enriched in tubulin and in spindle-associated proteins (see below) and when viewed in the light microscope spindles were the only structures seen (Fig. 3, *c* and *d*). Spindle isolation was monitored throughout by phase contrast microscopy (Fig. 3, *b* and *d*) and immunofluorescence microscopy with anti-tubulin (Fig. 3, *a* and *c*). The

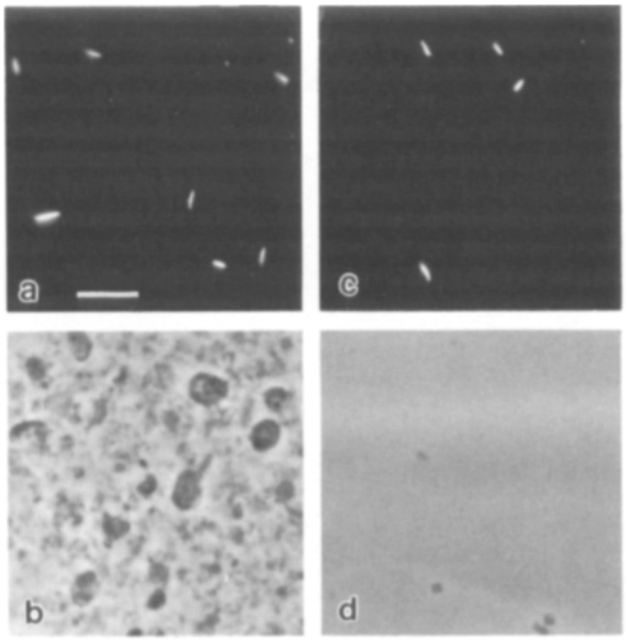

Figure 3. Fractions during spindle isolation. (*a* and *c*) Anti-tubulin immunofluorescence of a random microscope field from the beginning and end of the spindle isolation procedure. (*b* and *d*) Same fields as *a* and *c*, respectively, viewed with phase optics. (*a* and *b*) Filtered homogenate. (*c* and *d*) Isolated spindle fraction from the buffer/sucrose interface (see Materials and Methods). Bar, 10 μm.

filtered homogenate (see Materials and Methods) contained numerous chloroplasts, nuclei, and other organelles together with large numbers of spindles that were liberated from cells (Fig. 3, *a* and *b*). At this point there was a 40% reduction

Table I. Functional Spindles Retained during Spindle Isolation

Fraction	Percent Spindles Retained	ATP-Dependent Elongation* (percent control‡)
Filtered homogenate	100	63
2,600 *g* supernatant	60	39
Filtration retentate	30	39
Spindle fraction	30	39

* Fractions were incubated in 1 mM ATP for 5 min and percent gaps were determined for 500 spindles in each fraction.
‡ An aliquot of cells from cultures used to isolate spindles was permeabilized and spindles reactivated in 1 mM ATP for 5 min. Percent reactivation was determined and this value was used as control.

in the number of spindles that could undergo ATP-dependent reactivation when compared to spindle reactivation in permeabilized cells (Table I). After pelleting chloroplasts and nuclei there was an additional 20% loss of function (Table I). However after this point, while spindles continued to increase in purity throughout detergent treatment, filtration, and concentration onto a sucrose cushion (Fig. 3, *c* and *d*), the percentage of functional spindles remained unchanged (Table I). Based on size ~40% of the spindle structures in the final fraction may be half spindles. This was also suggested by observations of thiophosphate incorporation in spindle midzones of isolated spindles. In one experiment (data not shown), ATPγS was included in the isolation medium before bead beating cells. In this experiment isolated spindles incorporated thiophosphate into the spindle midzone but not at the poles. However, the shortest spindles in the population showed thiophosphate incorporation only at one end. This suggests that the short spindle structures may be half spindles.

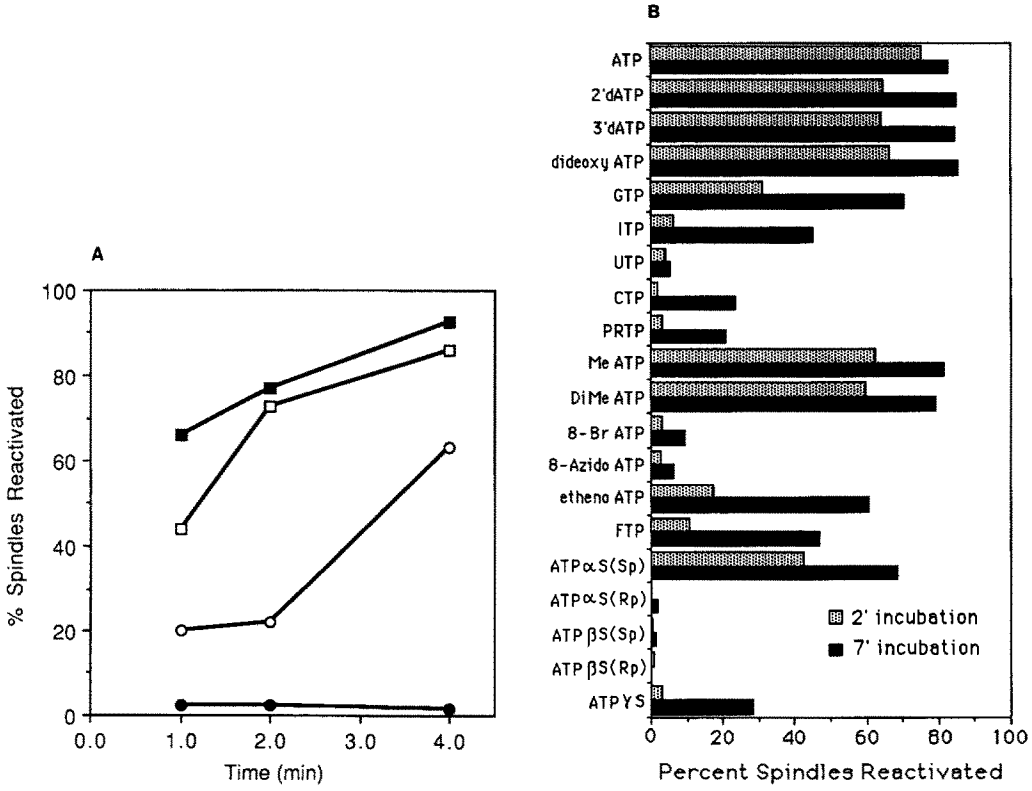

Figure 4. Effects of NTPs and ATP analogs on spindle elongation in vitro. (*a*) Permeabilized cells were incubated in ATP at concentrations of 1.0 (■), 0.1 (□), .01 (○), and .001 (●) mM for the times indicated. Populations of 250–500 spindles were then scored for spindle elongation by the formation of gaps. Spindle elongation showed a dependence on incubation time. (*b*) Each compound was incubated with permeabilized cells at 1 mM for either 2 min or 7 min. Full names of the compounds with the abbreviations used above are described in Shimizu et al. (1991).

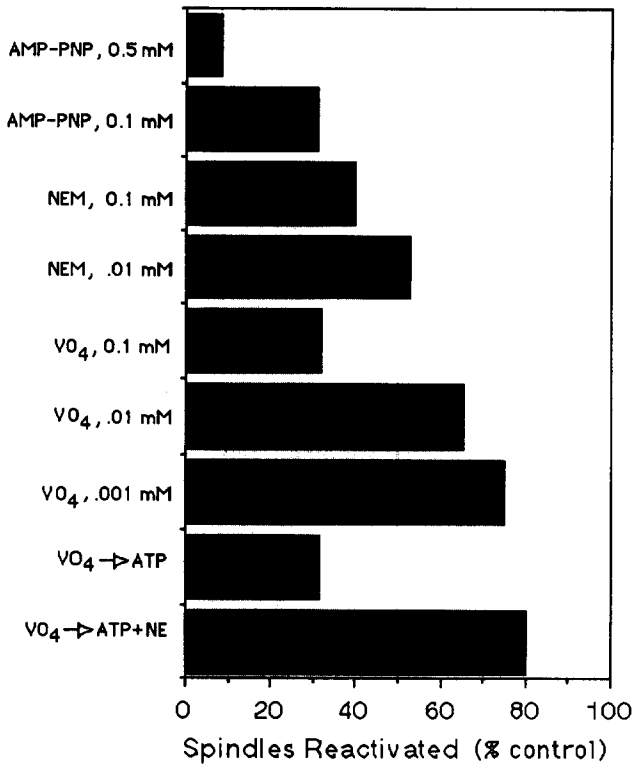

Figure 5. Effects of inhibitors on spindle elongation in vitro. Permeabilized cells were incubated for 5 min in 1 mM ATP plus the concentration indicated of 5′-adenylyl-imidodiphosphate (*AMP-PNP*), N-ethylmaleimide (*NEM*), or ortho-vanadate (VO_4). In the final two experiments listed, permeabilized cells were incubated for 5 min in 0.1 mM VO_4 alone, rinsed in PMEG, and then incubated in either 1 M ATP or 1 mM ATP and 0.5 mM norepinephrine (*NE*). Spindle reactivation was determined by the formation of gaps.

Nucleotide Specificity for Spindle Elongation

The degree to which ATP analogs support dynein- or kinesin-driven motility in vitro has recently been described and a profile of nucleotide utilization developed that clearly distinguishes these two ATPases from each other and from myosin (Shimizu et al., 1991). To ascertain which type of MT-based motor may be involved in spindle elongation, the relative abilities of NTPs and various ATP analogs to support spindle reactivation were measured using the gap assay. The percentage of gaps observed after ATP addition depended on time of ATP incubation and ATP concentration (Fig. 4 *a*). Thus the gap assay is sensitive to rates of spindle elongation. By using the gap assay at two time points the extent of spindle elongation supported by other NTPs and ATP analogs relative to ATP was determined. Permeabilized cells were incubated in the compounds listed in Fig. 4 *b* for either 2 or 7 min. Normally, spindle reactivation in 1 mM ATP is completed in 90% of the functional spindles by 2 min. GTP supported spindle elongation at roughly 50% the rate seen with ATP. ITP, UTP, and CTP all supported spindle elongation, although at very low rates. The deoxy-ATPs as well as methyl ATP and dimethyl ATP supported spindle elongation at rates similar to those seen in ATP. Low rates of elongation were seen with 8-bromo ATP, 8-azido-ATP, and purine riboside triphosphate. These results are consistent with kinesin-supported motility and inconsistent with dynein-supported motility.

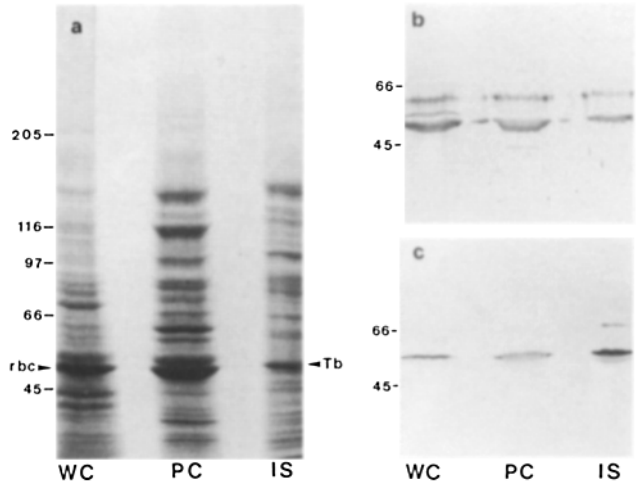

Figure 6. Polypeptides retained and lost in spindle-enriched fractions. (*a*) Coomassie-stained 4–10% SDS-PAGE of whole cell extract (*WC*), permeabilized cell extract (*PC*), and isolated spindle extract (*IS*). Molecular weight markers are indicated in kD. The major polypeptide present in WC and PC is the large subunit of ribulose-1,5-bisphosphate carboxylase (*RBC*). However in the IS RBC is greatly diminished and tubulin (*Tb*), which migrates slightly slower than RBC, is more prominent. (*b* and *c*) Immunoblots of the same fractions using anti-RBC (*b*) and anti-Tb (*c*) as probes show that RBC is reduced while Tb is enriched during preparation of permeabilized cells or isolated spindles. The top band in *b* represents a non-specific cross-reaction seen in every gel lane, including the molecular weight markers. 25–30 μg protein were loaded into each lane (*a*, *b*, and *c*). Molecular weights are marked in kD.

Inhibition of ATP-dependent Spindle Elongation

To further characterize the motor involved in spindle elongation in vitro, inhibitors of dynein and kinesin were assayed for their effects on spindle elongation. Vanadate has a high affinity for the γ-phosphate site in the dynein ATPase site and can form a dynein-ADP-vanadate complex. Vanadate is a potent inhibitor of dynein ATPase and motility by forming this dead-end kinetic block. At higher concentrations vanadate is a noncompetitive inhibitor of kinesin-driven MT translocation (Cohn et al., 1989). Spindles incubated in vanadate at concentrations of 100, 10, and 1 μM showed 32, 65, and 74% spindle elongation compared to controls, respectively, as measured by the gap assay. Vanadate inhibition of gap formation could be reversed by including 1 mM norepinephrine in the ATP-containing reactivation buffer subsequent to vanadate incubation (Fig. 5). *N*-ethyl-maleimide (NEM) acts as an alkylating agent that reacts with sulfhydryl groups of many polypeptides, but dynein's ability to function as a motor is especially susceptible to low levels (Gibbons and Gibbons, 1972). In contrast kinesin is rela-

Table II. Purification and Yield of Spindle Tubulin

Fraction	Volume	Total protein	Tubulin	Total tubulin	Tubulin/ protein	Yield
	(*ml*)	(*mg/ml*)	(*mg/ml*)	(*mg*)		
WC	155	6.5	.03	4.7	.005	1.00
PC	24	3.1	.02	.55	.008	.12
IS	10	.75	.04	.36	.048	.08

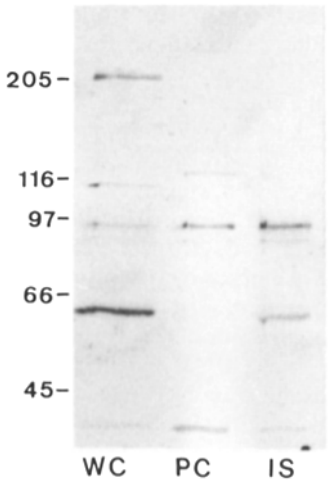

Figure 7. Immunoblot of whole cell (*WC*), permeabilized cell (*PC*), and isolated spindle (*IS*) extracts from *C. fusiformis* probed with anti-LAGSE. In enriched spindle fractions (*PC* and *IS*) anti-LAGSE cross-reacted predominantly with a polypeptide at ~95 kD, while this polypeptide is only slightly detectable in WC. 25–30 μg protein were loaded into each lane. Molecular weights are marked in kD.

tively insensitive to these levels of NEM (Vale et al., 1985; Cohn et al., 1989). Spindle elongation was inhibited by low concentrations of NEM. Coincubation of spindles in 10 μM NEM and 1 mM ATP allowed only 53% of the spindles to function (Fig. 5). The most potent inhibitor of functional central spindles was the nonhydrolyzable ATP analog 5'-adenylylimidodiphosphate (AMP-PNP). Kinesin-mediated MT transport is severely inhibited by AMP-PNP (Vale et al., 1985). Fig. 5 shows that only 31% spindles would reactivate relative to control spindles when a ratio of 0.1 mM AMP-PNP/1 mM ATP was applied to permeabilized cells while a 1:2 ratio rendered only 8% spindles reactivatable.

Polypeptides Present in Permeabilized Cell and Isolated Spindle Extracts

Polypeptides present in synchronized WCs were compared to those found in both PCs and isolated spindles (ISs) (Fig. 6 *a*). The polypeptide pattern was complex in all cases. However certain polypeptides were enriched in PC and IS fractions compared with WC fractions. The major protein component of both WC and PC was ribulose-1,5-bisphosphate carboxylase (RBC). Fig. 6 *b* shows that RBC was almost depleted in IS and reduced in PC. At the same time tubulin, presumably the major spindle component, increased in the PC and IS fractions (Fig. 6 *c*, Table II). In IS, tubulin was a major band that represented >5% of the total protein (Fig. 6, *a* and *c*, Table II).

Since PC and IS fractions both include functional spindles, other components required for spindle elongation are likely to comprise a subset of the enriched polypeptides in the PC and IS fractions. To demonstrate this each of the fractions was probed with a peptide antibody directed against a portion of the kinesin motor domain (Sawin et al., 1992). The peptide sequence recognized by this antibody is LNLVDLAGSE (LAGSE) and is conserved among members of the kinesin superfamily. Anti-LAGSE blocks *C. fusiformis* spindle elongation in vitro and cross reacts with a 95-kD polypeptide in IS that binds and releases MTs in a nucleotide-dependent fashion identical to that of kinesin (Hogan et al., manuscript submitted for publication). In WC the 95-kD polypeptide was not detected with anti-LAGSE (Fig. 7). However, as with tubulin (Fig. 6 *c*), the LAGSE-containing polypeptide at 95 kD was enriched during spindle isolation so that PC and, to a greater extent, IS showed significant cross reaction with anti-LAGSE (Fig. 7).

Discussion

For over 100 years, the exquisite architecture of the spindle, its assembly during prophase, and the dynamic events that occur during anaphase have drawn many to study mitosis. However, the complexity of the spindle apparatus with respect to morphology, polypeptide composition, variety of chromosome movements, and interactions with other cytoplasmic components have hindered our understanding of spindle function. In an attempt to minimize these complexities we have dissected the anaphase B machinery from cytoplasmic constituents as well as from those components of the spindle involved in anaphase A. The results presented here describe a new functional model where anaphase B movements can be studied in vitro in either isolated spindles or spindles contained in detergent permeabilized cells from the pennate diatom *C. fusiformis*.

The diatom central spindle is an excellent system to study the mechanism of spindle elongation. In diatoms, like many lower eukaryotes, the MTs of the central spindle are only involved in anaphase B movements and are spatially segregated from anaphase A components (Pickett-Heaps and Tippit, 1978; McDonald and Cande, 1989). Thus, nature, in the form of the diatom central spindle, has already contributed a crucial first step in purifying the anaphase B machinery. Also a high degree of structural order lends relative ease to interpretation of MT rearrangements that occur during spindle elongation both in vivo (Pickett-Heaps and Tippit, 1978; McDonald and Cande, 1989) and in vitro (Cande and McDonald, 1986) and has allowed us to define the primary mechanochemical event responsible for spindle elongation as the sliding apart of the two half spindles (Cande and McDonald, 1985).

The Gap Assay Is a Measure of Spindles That Have Elongated

When spindles are incubated in biotinylated tubulin before ATP addition, labeled tubulin is incorporated as new MTs nucleated at the poles and into the spindle midzone as two bands that flank the original zone of MT overlap. Thus this new tubulin is a marker for the free ends of the original half-spindle MTs. After ATP addition the spindle elongates, and the two bands in the midzone become one. The changes in distribution of newly incorporated tubulin in *C. fusiformis* spindles demonstrate that these spindles undergo an ATP-dependent rearrangement of MTs in the zone of MT overlap by the same mechanism as previously described for *S. turris* and fission yeast spindles (Cande and McDonald, 1985; Masuda et al., 1990). Although it is not possible to directly monitor MT overlap zone behavior these MT rearrangements indicate that antiparallel MTs must slide past one another in opposite directions through the zone of MT overlap.

As was found in the diatom *S. turris* (Cande and McDonald, 1986), ATP-induced gaps between half-spindles accurately identify spindles that have elongated. After ATP addition in the absence of exogenous tubulin a gap develops between the two half-spindles. The same proportion of spindles exhibit ATP-dependent sliding when half-spindle ends are marked with biotinylated tubulin.

The gap assay is an "all or nothing" measure of spindle elongation. However, individual spindles must vary in the time required to produce a gap in ATP. This could be due to differences in the length of the MT overlap zone or in the

rates of MT translocation in the overlap zone. This variation allows determination of the effect of a substrate on relative spindle elongation rate by varying concentration of nucleotide or time of incubation and counting percent spindles with gaps. The rate of spindle elongation in *C. fusiformis* is dependent on both ATP concentration (over a range of 0.05 to 1.0 mM) and time of incubation in ATP. The rate of spindle elongation in *S. turris* is also dependent on ATP concentration over the same ATP range (Masuda and Cande, 1987). Thus the relative rate of spindle elongation is accurately reflected by the ratio of spindles that form gaps at 2 min vs. 7 min.

Nucleotide Specificity for Spindle Elongation Is Consistent with Kinesin-mediated Motility

Relative rates of elongation in several NTP substrates were measured by holding substrate concentration constant at 1 mM and counting percent gaps at two time points. ATP is by far the best substrate for supporting spindle elongation. GTP supports reactivation but only at roughly half the relative rate of ATP. Low rates of spindle elongation were seen when ITP, UTP, and CTP were used as substrates.

Nucleotide support of spindle elongation in other anaphase B models is different. In permeabilized cell models of mammalian tissue culture cells (Cande, 1982) and fission yeast (Masuda et al., 1990) and in isolated spindles of *S. turris* (Cande and McDonald, 1986) only ATP will support anaphase B movement while in isolated sea urchin spindles pole to pole separation is supported equally well with either ATP or GTP (Rebhun and Palazzo, 1988). The explanation for the apparent differences in substrate specificity is unknown. However the relatively short MT overlap zone together with the extraordinary functional stability of *C. fusiformis* spindles may have allowed for more sensitive detection of slow elongation rates.

Remarkably, the relative rates of *C. fusiformis* spindle elongation supported by the NTPs tested are similar to those for MT translocation by kinesin in vitro with these substrates. Cohn et al. (1989) found the translocation rate of MTs on coverslips adsorbed with kinesin for 10 mM concentrations of GTP, ITP, UTP, and CTP to be 0.78, 0.27, 0.38, and 0.31 the rate, respectively, of ATP. Similarly 1 mM concentrations of these NTPs support MT translocation at rates 0.51, 0.33, 0.11, and 0.24, respectively, of those seen with 1 mM ATP (Shimizu, T., and R. D. Vale, unpublished results). Although precise rate determinations were not made for spindle elongation, the pattern of support for spindle elongation by various NTPs reflects that seen for MTs moving on kinesin.

Using a battery of ATP analogs Shimizu et al. (1991) have established characteristic signatures for dynein, kinesin, and myosin ATPase activity and motility rates in vitro. Schliwa et al. (1991) subsequently used the same ATP analogs to convincingly show that particle movement in both directions along MTs of *Reticulomyxa* in vitro closely match the ATP analog profile for dynein. ATP analog profiles for the rate of spindle elongation in vitro are very similar to those for kinesin and unlike those seen for dynein or myosin. Particularly diagnostic are dimethyl ATP and etheno ATP that support spindle elongation and kinesin motility at medium to high rates while supporting dynein motility poorly, if at all. Also, GTP, ITP, 8-bromo ATP, and purine riboside triphosphate support spindle elongation and kinesin motility, but do not support dynein motility. Thus both the spindle elongation motor(s) and kinesin are similar in that they are more promiscuous than dynein in the NTPs as well as the ATP analogs that can support motility.

The pharmacology of spindle elongation is similar in vitro in fission yeast, *S. turris*, *C. fusiformis*, and mammalian cells (Masuda et al., 1990; Cande and McDonald, 1986; Cande, 1982) suggesting that a related force generating system is used in all eukaryotic cells. Spindle elongation in vitro in *C. fusiformis* is at least partially blocked by inhibitors that affect dynein- and kinesin-based motility. The most dramatic effect is seen with AMP-PNP, an inhibitor of kinesin but not dynein based movements (Vale et al., 1985), where a 1:10 ratio with ATP inhibited spindle function by ~70%. However, inhibitors of dynein-based motility also blocked spindle reactivation. Spindle elongation in vitro in *C. fusiformis*, *S. turris* (Cande and McDonald, 1986), and lysed mammalian cells (Cande, 1982), is sensitive to vanadate in the 10 μM concentration range. In this range vanadate has little affect on squid kinesin motility (Vale et al., 1985). It should be noted, however, that 10 μM vanadate had significantly less affect on spindle elongation in the *C. fusiformis* model than in the *S. turris* or mammalian models. Although NEM is a general alkylating agent, dynein ATPases but not kinesin, are especially susceptible to inhibition at low concentrations (Gibbons and Gibbons, 1972). Spindle elongation in mammalian cells, diatoms and fission yeast is impaired by concentrations of NEM that do not affect kinesin-based motility (Cande, 1982; Cande and McDonald, 1986; Masuda et al., 1990). Only one kinesin-related protein, the ncd motor protein, has been characterized with respect to inhibitors and it also shows some unusual features (Walker et al., 1990). Microtubule gliding in vitro supported by ncd is sensitive to NEM, but is not affected by AMP-PNP, except at high concentrations. These inhibitor studies underscore the fact that the pharmacology of spindle elongation in vitro has some novel features; perhaps reflecting a motor protein(s) that has not been identified biochemically in other systems.

Purity and Polypeptide Composition of Spindle Preparations

Since the 1950s several groups have made spindle-enriched preparations from various organisms with the goals of characterizing anaphase chromosome movements and identifying spindle-associated proteins by biochemical procedures (reviewed in Cande, 1989). Thus far, no spindle model has proved satisfactory for both purposes (Pratt et al., 1980; Leslie and Wilson, 1989; Dinsmore and Sloboda, 1988; Rebhun and Palazzo, 1988; Palazzo et al., 1991). The permeabilized cells and isolated spindles described here have two attributes that separate them from other spindle preparations. First, the majority of spindles in these fractions retain the ability to elongate in vitro. Second, over 0.4 mg spindle protein can be obtained routinely several times per week. Using isolated *C. fusiformis* spindles it may now be possible to assign functions to specific polypeptides by using functional assays in concert with biochemical isolation procedures.

The presence of RBC was used as a measure of cytoplasmic contamination of our spindle preparations because it is by far the most abundant protein in whole cells. This represents as much as 20% of the soluble protein pool in many algal cells (Plumley et al., 1986). There is a dramatic

decrease in RBC with a concomitant increase in tubulin as a percentage of total protein during spindle isolation, indicating a substantial enrichment for spindles. Tubulin is one of the major bands that stain by Coomassie blue in the isolated spindle fraction. However some RBC remains even in the isolated spindle fraction indicating that contamination by cytoplasmic proteins is still a problem. A major contaminant that persists in the preparation throughout the isolation procedure is a "matrix", possibly an extracellular mucopolysaccharide that helps diatoms adhere to surfaces. This matrix may nonspecifically bind soluble proteins and carry them along through spindle purification.

The spindle isolation procedure described yields a tenfold enrichment of tubulin compared to tubulin in whole cell extracts. However, this is an underestimate of enrichment of spindle proteins. Presently, tubulin is ~5% of the total protein in the isolated spindle fraction and 0.5% of the protein in whole cell extracts. Since most soluble protein is filtered out during the isolation procedure the tubulin present in the purified spindle fraction represents mostly spindle tubulin. However, the whole cell extracts contain a large tubulin pool derived from depolymerized interphase MT arrays. Preliminary EM studies suggest that the average *C. fusiformis* spindle contains about 20 MTs that are ~4-μm long. This is ~4 $\times$ 10^{-8} μg of tubulin per spindle. Under present growth conditions, minimal cell density at harvest is 1.5 $\times$ 10^9 cells/liter with a minimum mitotic synchrony of 33%. We calculate that the cells derived from one bulk harvest of 80 liters should contain ~1.6 mg spindle tubulin. Based on this calculation, rather than the measured tubulin concentration, we may have achieved as much as a 40-fold enrichment of spindle protein in our isolated spindle fraction with an estimated yield of ~25%.

Identification of a Putative Spindle-associated Motor

During spindle isolation enrichment was demonstrated for a possible kinesin-related protein. Anti-LAGSE was raised against a 10 amino acid sequence conserved among members of the kinesin superfamily; it and a similar peptide antibody have been used successfully to identify kinesin related proteins in extracts from *Xenopus*, CHO cells (Sawin et al., 1992), and sea urchins (Cole et al., 1992). Anti-LAGSE blocks spindle elongation in the permeabilized cell model and function can be rescued with the LAGSE peptide (Hogan et al., manuscript submitted for publication). Anti-LAGSE also identified a polypeptide of ~95 kD in IS that binds to MTs in the presence of AMP-PNP and is released from MTs with ATP (Hogan et al., manuscript submitted for publication). This is characteristic of the nucleotide-dependent MT-binding behavior of kinesin. Since this polypeptide is enriched in IS, is the major polypeptide identified by anti-LAGSE, and function in the in vitro model is blocked by anti-LAGSE, it is an excellent candidate for a spindle motor. The IS fraction should be a useful starting point for the biochemical purification of the 95-kD polypeptide.

C. fusiformis has provided a system where reliable pharmacological parameters of spindle elongation enzymology can be measured in vitro. This system also provides a bridge between in vitro analysis of spindle elongation and biochemical dissection and analysis of the components involved. At present, the isolated diatom central spindle remains the only preparation where it is possible to analyze in vitro the mechanism of antiparallel MT sliding during spindle elongation with morphological and biochemical techniques and relate these results to what we know about anaphase B in vivo.

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