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Inhibition of anaphase spindle elongation in vitro by a peptide antibody that recognizes kinesin motor domain

(mitosis/anaphase B/kinesin-related proteins)

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ABSTRACT Isolated central spindles or spindles in detergent-permeabilized cells from the diatom Cylindrotheca fusiformis can undergo ATP-dependent reactivation of spindle elongation in vitro. We have used a peptide antibody raised against a 10-amino acid portion common to the kinesin superfamily motor domain to look for kinesin-like motor activity during anaphase B of mitosis. The peptide antibody localizes to central spindles. Upon ATP reactivation of spindle elongation, antigens recognized by the antibody are associated exclusively with the central spindle midzone where antiparallel microtubules of each half-spindle overlap. The antibody recognizes several polypeptides by immunoblot using isolated spindle extracts. One of these polypeptides behaves like kinesin with respect to nucleotide-specific binding to and release from taxol-stabilized microtubules. Precipitation of the spindle model with the peptide antibody inhibits subsequent ATP reactivation of spindle elongation. Coincubation of the peptide antibody with peptide antigen rescues spindle function. These results support a role for kinesin-related protein(s) in spindle elongation (anaphase B) of mitosis and suggest that one or several polypeptides that we have identified in spindle extracts may fulfill this function.

Diatom spindles are important model systems for describing the rearrangements of microtubules (MTs) that occur during anaphase because the MTs associated with spindle elongation are closely packed in an antiparallel overlapping MT array (the central spindle) and are spatially separated from kinetochore and astral MTs (1, 2). Using functional spindles prepared from diatoms we have demonstrated that forces sufficient to drive half-spindles apart during spindle elongation (anaphase B) must be generated in the zone of antiparallel microtubule overlap (3–5). Similar forces generated by mechanochemical interactions in the zone of microtubule overlap may play a role during mitosis in most eukaryotic cells (6–9). The penate diatom Cylindrotheca fusiformis can be readily grown in bulk cultures suitable for biochemistry and the cells can be arrested in mitosis by a combination of light/dark cell cycle synchronization and drug arrest. At harvest, 40–60% of the cells contain 4- to 5-μm-long metaphase/early anaphase spindles and interphase MT arrays are absent (10). After isolation or cell permeabilization the two overlapping half-spindles that comprise the central spindle retain the ability to slide apart when incubated with 1 mM MgATP. ATP-dependent reactivation of spindles in vitro leads to spindle elongation. The resulting rearrangement of MTs in the zone of microtubule overlap leads to the formation of a bend or a gap between the two half-spindles bridged by only a few MTs (3, 10).

Although inhibitors of dynein- and kinesin-mediated motility inhibit spindle elongation in vitro (11, 12), the profile of nucleotides and ATP analogs that support spindle elongation in vitro (10) more closely resembles that found for conventional kinesin-based motility (13). However, antibodies prepared against conventional kinesins (or dyneins) have failed to localize to diatom spindles or to cross-react on immunoblots of isolated diatom spindles (unpublished data). Thus, these and other immunolocalization experiments (e.g., ref. 14) provide no evidence that conventional kinesin is a motor for spindle elongation.

Kinesin heavy chain (KHC) is a member of a superfamily of proteins that also includes numerous kinesin-related proteins (KRPs) identified at the nucleic acid level by genetics or PCR (15–19, 20, 21) and at the protein level using peptide antibodies (22, 23). In an attempt to identify possible KRPs in diatom spindles we used antibodies generated against short peptide stretches located in the kinesin motor domain that share sequence identity with all known KRPs (20, 21). One antibody that cross-reacts by immunoblotting with kinesin as well as with KRPs (22) recognized diatom central spindle components. The peptide sequence recognized by the antibody is LNLVDLAGSE (amino acid region 226–235 of KHC (14)) and we refer to this as the “LAGSE” peptide (22). This antibody recognizes KRPs in mammalian and frog cells but can also cross-react with some non-KRPs in crude extracts. Subsequently, this antibody was used to probe for KRP motor activity in the diatom spindle by assaying the antibody’s ability to block in vitro spindle elongation. The results obtained suggest that KRP(s) may be responsible for spindle elongation in vitro and presumably contribute to spindle elongation forces in vivo, in diatoms as well as other organisms.

MATERIALS AND METHODS

Cultures of C. fusiformis were seeded into sea water containing 1/2 medium (24) and synchronized by a light/dark cycle as described (10). Metaphase spindles were collected from synchronously dividing cells in 0.1 μM nocodazole and harvested by tangential flow filtration. At this drug concentration interphase microtubule arrays are disrupted but spindles continue to form and are arrested at metaphase (10). Cells were permeabilized by gentle shaking on ice for 15 min in PMEG (50 mM Pipes [pH 7.0], 5 mM MgSO4, 5 mM EGTA, 40 mM β-glycerophosphate, 100 μM Trolox, 1 mM dithiothreitol, a protease inhibitor cocktail (3, 12), and 1 mM

Abbreviations: MT, microtubule; KHC, kinesin heavy chain; KRP, kinesin-related protein; AMP-PNP, adenosyl-imidodiphosphate; IS, isolated spindle extract.

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phenylmethylsulfonyl fluoride) with 1% Triton X-100 and 3% dimethyl sulfoxide added. Permeabilized cells were pelleted 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 PMEG to wash Triton X-100 and dimethyl sulfoxide from cells.

For experiments involving in vitro reactivation of spindle elongation and for immunolocalization of spindles or spindle-associated antigens, small aliquots of the permeabilized cell suspension were spun onto coverslips through PMEG. For immunolocalization, coverslips were fixed in cold methanol and processed as described (10) with antibody against tubulin or LAGSE [anti-LAGSE and anti-HIPRESKLT] were generated and affinity purified against immobilized peptide as described (22). For reactivation of spindle elongation, permeabilized cells on coverslips were incubated in 1 mM ATP for 5 min at 23°C before fixation. To test for antibody inhibition of spindle elongation 20 μl PMEG, 150 μg of antibody per ml in PMEG, or 150 μg of anti-LAGSE per ml plus a 10-fold molar excess of peptide in PMEG were incubated on coverslips for 15 min at 24°C (preincubation concentrations of anti-LAGSE were as shown in Fig. 3b). Coverslips were then washed in PMEG and incubated 5 min with 1 mM ATP in PMEG before being fixed and processed for anti-tubulin immunofluorescence as above. Spindles from Stephanopyxis turris were isolated onto coverslips and reactivated in 1 mM ATP for 5 min as described (3). Spindles were fixed in cold methanol as described above and then labeled sequentially with antibodies to LAGSE and tubulin, respectively. Double labeling was done with fluorescein isothiocyanate anti-rabbit IgG (LAGSE) and rhodamine antimouse IgG (tubulin).

Isolated spindle extract (IS) was made as described (10). Briefly, 90-liter synchronized cell cultures were concentrated to 250 ml and homogenized in PMEG containing 3% dimethyl sulfoxide by bead beating with 0.1-mm glass beads. Glass cell walls were spun out of the homogenate at 200 × g and the homogenate was then spun at 2600 × g, 30 min, 4°C. The resulting supernatant was made 1% Triton X-100 and subjected to tangential flow filtration to eliminate soluble proteins. Finally, isolated spindles were collected on a 20% sucrose cushion at 8000 × g, 10 min, 4°C and extracted with 0.5 M NaCl; the extract was dialyzed against PMEG. The KRP-enriched MTs from sea urchin eggs (K-MT) were prepared in the presence of adenylyl-imidophosphate (AMP-PNP) as described (23).

For MT copelleting experiments, 80 μl of bovine neurotubulin (20 mg/ml) that had been purified by three cycles of polymerization/depolymerization and DEAE chromatography was polymerized at 22°C in the presence of 20 μM taxol and 2 mM GTP. IS was prepared from 90 liters of diatoms and the resulting volume was 1 ml after protein aggregates had been eliminated by centrifuging at 4°C, 30 min, 50,000 rpm in a TLA 100.3 rotor (Beckman). Before adding MTs, IS was made 20 μM taxol, 1 mM GTP, and 2 mM AMP-PNP. MTs were added to IS and the mixture was incubated at 22°C, 30 min before dividing the mixture into three equal volumes and pelleting MTs through a 1-ml 60% glyceral cushion, 30 min, 22°C, 70,000 rpm in a TLA 100.3 rotor. The resulting supernatant was saved (AMP-PNP, S1). The pellets were washed with PME buffer and one was saved (AMP-PNP, P1) while the remaining two pellets were resuspended in PME buffer, 20 μM taxol, and 1 mM GTP. One suspension was made 2 mM AMP-PNP and the other was made 10 mM MgATP. Each treatment was incubated at 22°C, 30 min, and then centrifuged as before. Supernatants and pellets of the treatments were saved (AMP-PNP, S2 and P2, and ATP, S and P, respectively). Each supernatant and pellet fraction was split into two equal volumes and loaded onto duplicate 4–10% SDS/polyacrylamide gels. After electrophoresis one gel was stained with Coomassie blue and the other was used for immunoblotting. Gels were run and immunoblotting was performed as described (10).

RESULTS

After cell permeabilization (Fig. 1a) or spindle isolation (not shown) central spindles of C. fusiformis appeared as a bright bar 4–5 μm in length when labeled with anti-tubulin. As shown previously (3) and here (Fig. 1a) using large spindles from the diatom S. turris, in vitro reactivation of spindle elongation in C. fusiformis with ATP caused half-spindles to slide apart until a gap formed between the two half-spindles (Fig. 1b). Determining the percentage of gaps in a population of C. fusiformis spindles can be done quickly and unambiguously and it accurately reflects the percentage of spindles that have elongated (10).

Anti-LAGSE localized to antigens in mitotic spindles of permeabilized C. fusiformis cells (Fig. 1c and d). Some cytoplasmic staining was also observed. Staining was seen throughout the length of spindles and in many cases appeared biased toward the spindle midzone. To clearly demonstrate the location of antigen recognized by anti-LAGSE before and

**Fig. 1.** Reactivation of spindle elongation in vitro and staining of spindles with anti-LAGSE. (a and b) Anti-tubulin staining of the central spindle in permeabilized C. fusiformis cells before (a) and after (b) incubation in 1 mM ATP for 5 min. After ATP addition the two half-spindles slide apart and a gap develops in the spindle midzone (arrow in b). It has been shown in this (10) and other in vitro spindle elongation models (3) that gap formation between the two half-spindles after ATP incubation reflects structural changes associated with antiparallel microtubules sliding apart in opposite directions. (c) Anti-LAGSE also cross-reacts with C. fusiformis spindles (arrow). Anti-HIPRESKLT staining is indistinguishable from anti-LAGSE (not shown). (d) 4'-6-Diamidino-2-phenylindole staining of c showing chromosomes were in the process of karyokinesis. (e and f) Anti-LAGSE (e) and anti-tubulin (f) staining of an isolated spindle from S. turris. (g and h) Anti-LAGSE (g) and anti-tubulin (h) staining of an S. turris spindle after reactivation of spindle elongation. Following ATP reactivation of spindle elongation the LAGSE-containing antigen(s) is localized only at the spindle midzone. (Bar = 5 μm.)
after ATP reactivation of spindle elongation in vitro, large spindles isolated from the diatom S. turris (2) were used (Fig. 1 e–h). After spindle reactivation the antigen was no longer distributed uniformly throughout the spindle but was concentrated in the spindle midzone and to a lesser extent at the poles (pole staining not seen in the particular spindle shown in Fig. 1g). Antigen redistribution and/or differential extraction of antigen off non-overlapping MTs by the MgATP-containing buffer could account for this drastic change in localization pattern. The same anti-LAGSE localization pattern was apparent in C. fusiformis spindles but the much larger spindles of S. turris, that contain >30 times more MTs than C. fusiformis spindles, most clearly demonstrate the change of LAGSE antigen distribution in these two diatoms.

The antibody consistently recognized polypeptides in the 60- to 100-kDa range on immunoblots of IS from C. fusiformis (Fig. 2). The bands recognized in this molecular mass range were also enriched manyfold when compared to whole cell extracts (10). Most kinesin-like genes identified thus far have predicted protein products in the range of 70–130 kDa (15–19). Anti-LAGSE recognizes a set of polypeptides from sea urchin egg extract that were identified by another, similar peptide antibody (23) (Fig. 2a, K-MT). The cross-reacting bands at 95 and 85 kDa in sea urchin extracts have been shown to be KRP s that behave biochemically like KHC (23). In addition, the cross-reacting sea urchin polypeptides that have so far been analyzed at the DNA level appear to contain the kinesin motor domain sequence (unpublished results). IS also contains a polypeptide of ~95 kDa that cross-reacts strongly with anti-LAGSE (Fig. 2a, IS) and may therefore be a bona fide KRP.

KHC will bind to MTs in the presence of AMP-PNP (a nonhydrolyzable analog of ATP) and can be released from MTs with ATP (23). When spindle-enriched diatom extracts were incubated with exonuclease-taxed-stabilized MTs in the presence of AMP-PNP, many of the polypeptides that cross-react with anti-LAGSE copelleted with the MTs upon centrifugation (Fig. 2b, blot, AMP-PNP, P). Subsequent resuspension of the pellet followed by incubation with MgATP caused much of the 95-kDa polypeptide to be released from the MTs (Fig. 2b, blot, ATP, S). The reason for incomplete release of the 95-kDa polypeptide after ATP treatment is unknown, but these proteins could possess nucleotide-independent MT binding sites that would not allow their release from MTs under the conditions used. However, if the resuspended pellet was instead incubated with AMP-PNP again, all of the 95-kDa polypeptide identified by immunoblotting with anti-LAGSE pelleted with the MTs (Fig. 2h, blot, AMP-PNP, P). Thus diatom spindle extracts contain several potential KRPs based on anti-LAGSE reactivity and MT copelleting in the presence of AMP-PNP. The most prominent of these are 60, 95, and 100 kDa, respectively. In addition, the 95-kDa polypeptide exhibits nucleotide requirements similar to KHC for release from MTs, although the 60- and 100-kDa polypeptides did not show this behavior. These data indicate that with a high rate of success anti-LAGSE is able to identify KRPs (as seen in AMP-PNP MT pellets from sea urchin extracts) and that diatom spindle extracts potentially contain several KRPs.

To examine the functional consequence of antibody binding on spindle elongation in vitro, we preincubated permeabilized C. fusiformis cells in antibody prior to spindle reactivation by ATP. Spindle elongation was subsequently scored by the appearance of gaps between half-spindles. When spindles were preincubated in anti-LAGSE at 150 μg/ml, ~90% of the spindles failed to reactivate (Fig. 3). However, spindles preincubated with an antibody against another conserved peptide in the kinesin motor domain [HlPRESKL (amino acids 274–283 of KHC (14))] at 600 μg/ml showed only a slight reduction in reactivation when compared to controls (Fig. 3). This peptide antibody also immunolocalizes to spindles in permeabilized diatom cells (not shown), although it does not react well with IS polypeptides on immunobLOTS. Spindles were also preincubated with the anti-tubulin used to stain spindles for the gap assay. Significantly weaker anti-tubulin staining subsequent to fixation and immunofluorescence processing confirmed that the antibody had bound to spindles during preincubation (not shown). As with anti-HlPRESKL, preincubation with anti-tubulin caused no significant inhibition of spindle elongation (Fig. 3a). These results suggest the inhibition of function is not due to nonspecific cross-linking of spindle components by IgG.

Inhibition was due to anti-LAGSE interaction with a spindle component(s) since spindle function could be rescued with the LAGSE peptide. When LAGSE peptide was included during preincubation with anti-LAGSE ~90% of the spindles could subsequently undergo ATP-dependent elon-

Fig. 2. Polypeptides identified by anti-LAGSE in diatom isolated spindle extracts (IS). Molecular masses are marked in kDa. (a) Coomassie-stained gel (GEL) and corresponding immunoblot (BLOT) probed with anti-LAGSE shows the antibody-bound KRPs in a sea urchin MT preparation done in the presence of AMP-PNP that were previously identified (K-MT) (23). In IS anti-LAGSE bound polypeptides are in the 60- to 100-kDa range. Of particular interest is a band of 95 kDa that comigrates with a KRP identified from sea urchin egg extract (23). (b) Coomassie-stained gel (GEL) and corresponding immunoblot (BLOT) probed with anti-LAGSE of IS polypeptides that copelleted with taxol-stabilized MTs. The tubulin used to make MTs was free of extraneous polypeptides (GEL, Tb) including any anti-LAGSE cross-reacting polypeptides (BLOT, Tb). Much of the 95-kDa polypeptide copelleted with MTs in the presence of 2 mM AMP-PNP (BLOT, AMP-PNP, P). When AMP-PNP, P was resuspended in the presence of 2 mM AMP-PNP and centrifuged, all of the 95-kDa polypeptide pelleted with the MTs (BLOT, AMP-PNP, P). However, when AMP-PNP, P was resuspended in the presence of 10 mM ATP, much of the 95-kDa polypeptide released from MTs and was found in the supernatant after centrifugation (BLOT, ATP, S).
DISCUSSION

In previous studies we have made use of the exquisite architecture of the diatom central spindle to demonstrate that the primary mechanochemical event involved in diatom spindle elongation is the sliding apart of the MTs of the two half-spidelles due to forces generated within the zone of MT overlap (3–5, 10). Similar experiments using cells of permeabilized fission yeast demonstrated that a sliding mechanism may be responsible for spindle elongation in this organism as well (6). Various other experiments including fluorescent recovery after photobleaching of fluorescently labeled MTs in the mammalian spindle midzone (9) and quantitative electron microscopy (25) are also consistent with a sliding mechanism. Thus, in nature, this may be a common mechanism that contributes to spindle elongation. Using a battery of ATP analogs, Shimizu et al. (13) have established characteristic rate signatures for various mechanochemical enzymes, including kinesin. Spindle elongation in vitro in C. fusciformis and kinesin-driven MT gliding are very similar with respect to rates of movement supported by ATP analogs and are unlike dynein-based motility (10). In experiments described here we have demonstrated that an antibody that recognizes KRP blocks spindle elongation in vitro. These two lines of evidence strongly suggest that spindle elongation in vitro and presumably in vivo is driven by KRP. Moreover, two KRP recently have been identified that are present in the spindle midzone of mammalian mitotic spindles during anaphase (26, 27) and one of them is capable of moving antiparallel MTs apart in vitro (26).

Previous isolated spindle (4, 10) and permeabilized cell (6, 10) models have shown that spindles can continue to elongate to lengths greater than the extent of the original zone of MT overlap in vitro by adding only purified tubulin [that polymerizes onto the free (+) ends of central spindle MTs] and ATP. This requires that molecular motors must somehow interact with antiparallel MTs in a MT overlap zone that consists entirely of exogenously added tubulin. These models predict that motor molecules should be retained in the spindle midzone during antiparallel MT sliding. In the present study anti-LAGSE localized to the entire spindle prior to ATP reactivation but only localized to antigens in the spindle midzone subsequent to in vitro spindle elongation. This could be due to either redistribution of antigen or extraction of antigen along non-overlapping MTs, or to a combination of both. Regardless of the cause these results are consistent with the observed behavior of several in vitro spindle models (4, 6, 10) and with the view that the motor(s) responsible for spindle elongation remains in situ in the zone of MT overlap during anaphase B. The nature of the motor’s retention in the spindle midzone remains unclear.

The direct demonstration that certain classes of mechanochemical enzymes are responsible for specific motility events in the spindle has lagged behind genetic predictions. Our strategy in these experiments was to use a functional model and a probe general enough to identify several members of the kinesin superfamily. Although the LAGSE sequence is clearly indicative of the kinesin superfamily and peptide antibodies against this sequence have successfully been used to identify KRP, other unrelated proteins contain portions of this sequence. In diatom whole cell extracts many polypeptides have been identified by anti-LAGSE. However, after spindle isolation only certain polypeptides were enriched that cross-reacted with anti-LAGSE (10).

Several of the polypeptides identified by anti-LAGSE in spindle-enriched fractions also exhibit MT binding in the presence of AMP-PNP and one (95 kDa) shows nucleotide-specific MT release typical of kinesin superfamily members. It is unlikely that polypeptides with no known MT association would behave in such a manner. As yet it is unclear whether the cross-reacting polypeptides in the spindle-enriched fraction are KRP and/or contribute to spindle elongation. Further work is necessary to show such a relationship. However the establishment that spindle elongation shows the enzymatic characteristics expected for a kinesin (10) together with evidence presented here that a general anti-KRP inhibitors spindle elongation strongly suggest that KRP actively participate in anaphase spindle elongation. Using function-blocking antibodies to correlate loss of spindle function with specific polypeptides eluted from spindles under various inactivating conditions should allow us to sort out specific
motor functions and to directly identify the motor(s) involved in spindle elongation.

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