Identification and Partial Characterization of Mitotic Centromere-associated Kinesin, a Kinesin-related Protein That Associates with Centromeres during Mitosis

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Abstract. Using antipeptide antibodies to conserved regions of the kinesin motor domain, we cloned a kinesin-related protein that associates with the centromere region of mitotic chromosomes. We call the protein MCAK, for mitotic centromere-associated kinesin. MCAK appears concentrated on centromeres at prophase and persists until telophase, after which time the localization disperses. It is found throughout the centromere region and between the kinetochore plates of isolated mitotic CHO chromosomes, in contrast to two other kinetochore-associated microtubule motors: cytoplasmic dynein and CENP-E (Yen et al., 1992), which are closer to the outer surface of the kinetochore plates. Sequence analysis shows MCAK to be a kinesin-related protein with the motor domain located in the center of the protein. It is 60-70% similar to kif2, a kinesin-related protein originally cloned from mouse brain with a centrally located motor domain (Aizawa et al., 1992). MCAK protein is present in interphase and mitotic CHO cells and is transcribed as a single 3.4-kb message.

THE cytoskeleton is intimately involved in the spatial organization of the cytoplasm in eukaryotic cells (Yisraeli et al., 1990; Wick, 1991; Fulton, 1993). Microtubules are a ubiquitous and fundamental component of the machinery for organelle movement. They serve as a framework for the transport and localization of subcellular components. Some of the credit for the proper sorting of diverse elements within the cell is being given to the growing family of kinesin-related microtubule-dependent motor molecules. The identification of so many different kinesin-related proteins in single organisms has led investigators to suggest that different kinesins are responsible for the transport of different cargoes within the cell, or that these different kinesins are subject to differential regulation (reviewed in Goldstein, 1993a). Tissue-specific and developmental stage-specific members of the kinesin superfamily have been identified (Stewart et al., 1991; Aizawa et al., 1992). Furthermore, there is a growing body of genetic evidence implicating different kinesin-like proteins in neuron-specific (Hall and Hedgecock, 1991) versus general mitotic functions (Saxton et al., 1991; Endow, 1993).

Members of the kinesin superfamily are defined by peptide sequence homology to the motor domain of kinesin heavy chain, the first member of the family to be identified (Brady,

1985; Vale et al., 1985a). Kinesin heavy chain was originally identified as a potential vesicle transporter in axons, a fairly specialized tissue type. However, although neuronal function and axon positioning are disturbed in kinesin heavy chain mutants, synaptic vesicle populations appear normal (Saxton et al., 1991; Gho et al., 1992). Therefore, it may be that kinesin heavy chain is not a specialized transporter of synaptic vesicles in neurons, but that it has a more general role involving membrane-bound organelles in all cells (Pfister et al., 1989; Hollenbeck and Swanson, 1990; Rothwell et al., 1993; Mitsui et al., 1993).

Slightly less functionally ambiguous is the genetic evidence for the specialized mitotic/meiotic kinesins such as bimC, klpA (*Aspergillus nidulans*; O'Connell et al., 1993), ncd, nod (*Drosophila melanogaster*; reviewed in Endow, 1992), KAR3, CIN8, KIP1, (*Saccharomyces cerevisiae*; Roof et al., 1992; Hoyt et al., 1993), and cut7 (*Schizosaccharomyces pombe*; Hagan and Yanagida, 1992). Mutations in these genes appear to be specific for maintaining spindle structure and/or the fidelity of chromosome segregation and, in the case of KAR3, nuclear migration (Meluh and Rose, 1990). Where available, immunolocalization studies show that these kinesin-related proteins associate with the mitotic spindle or structures therein (Hagan and Yanagida, 1992; Hatsumi and Endow, 1992; Hoyt et al., 1992; Theurkauf and Hawley, 1992). Although these kinesins have a role in a fundamental cellular process, functional redundancy within the system renders some of these genes dispensable (Goldstein, 1993b).

The globular motor domain of kinesin heavy chain converts the energy of ATP hydrolysis into movement along microtubules (Bloom et al., 1988; Scholey et al., 1989; Yang et al., 1990). Kinesin-related proteins have been identified that can move in either direction along a microtubule (McDonald et al., 1990; Walker et al., 1990); however, it appears that as yet undefined determinants within the motor domain structure may render the directionality uniform for any particular kinesin (Stewart et al., 1993). Presently, it has been shown that all plus end–directed kinesins have the motor domain near the NH_2 terminus of the peptide and all minus-end directed kinesins have COOH-terminal motor domains. Therefore, directionality is suggested, but not determined, by the location of the motor domain within the primary sequence of the kinesin peptide.

We have isolated a number of CHO cell kinesins in a screen using affinity-purified antipeptide sera raised against conserved regions of the kinesin motor domain (Sawin et al., 1992). In this report, we describe one of these clones which is interesting in several of these respects. It is associated with the centromeric region of mitotic chromosomes throughout mitosis. For this reason, we refer to it as MCAK[1] (mitotic centromere-associated kinesin). It has a centrally located motor domain, in contrast to most kinesins previously described. MCAK may be related to a recently described kinesin-like protein kif2 (Aizawa et al., 1992), which also has a centrally located motor domain. MCAK shares a high degree of identity with kif2. Finally, we have found a protein antigenically related to MCAK in all species tested so far.

Materials and Methods

Screening and Expression of MCAK

Affinity-purified rabbit polyclonal sera against two conserved regions of the kinesin motor domain were used in this study. The production and characterization of this sera has been described elsewhere (Sawin et al., 1992). Anti-LAGSE was used to screen a lambda Uni-Zap CHO cell cDNA library (Stratagene, La Jolla, CA). 17 positive clones were isolated from 600,000 plaques. Sequence analysis using ^{35}S-αdATP (New England Nuclear, Boston, MA) and Sequenase kit 2.0 (United States Biochemical Corp., Cleveland, OH) revealed seven of these to be kinesin-related proteins. Several of these clones were subcloned into the pMAL expression vector protein purification system (New England Biolabs Inc., Beverly, MA). Since this vector is available in only one reading frame and is only compatible with lambda gt11 clones, the other two reading frames were constructed by directionally incorporating a reading frame adapter and a SmaI-BamHI (Stratagene) adapter into the EcoRI and BamHI cloning sites:

pMAL-cRI 5'-gaattcggatcctct-3'
  (New England Biolabs)
pMAL-cRI2 5'-gAATTGGGAATTCCCCGGGGgatcctct-3'
pMAL-cRI3 5'-gAATTGGCGAATTCCCCGGGGgatcctct-3'

Some of the clones required pMAL-cRI3, and others required pMAL-cRI2 for in-frame expression. A restriction map of the longest MCAK clone revealed that it contained both an internal EcoRI and XhoI site. Therefore, the longest fragment of a partial digest of the clone was subcloned into the EcoRI and SalI sites of pMAL-cRI2. Expressed fusion protein was purified on an amylose column according to the New England Biolabs protocol. Sequencing of the full-length clone was done by primer extension on both strands on a sequencer (model 373A; Applied Biosystems, Inc., Foster City, CA) by the Bimolecular Resource Center DNA Sequencing Facility at the University of California at San Francisco.

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1. *Abbreviation used in this paper*: MCAK, mitotic centromere-associated kinesin.

PCR Cloning of MCAK 5' End

Nested PCR was performed with two sets of primers on the Stratagene CHO cDNA library. In each case, the 5'-primers were derived from the pBluescript II vector and the 3' primers were derived from the 5' end of the MCAK clone. Vent polymerase (New England Biolabs) was used for two sets of 15 and 25 cycles, respectively. A range of product sizes was obtained, the smallest corresponding to the 450-bp size of the known product. The PCR products were blotted onto nitrocellulose and probed with the first 5' 200 bp of the MCAK clone labeled with HRP using the enhanced chemiluminescence direct nucleic acid detection system (Amersham Corp., Arlington Heights, IL). All the PCR fragments hybridized to this clone, but control non-MCAK DNA did not. The products fortuitously contained an EcoRI site at either end of each fragment. The PCR products were digested with EcoRI, and the two largest fragments (~710 and 630 bp, 280 of which is known sequence from the 5' end of MCAK) were gel purified and subcloned into pBluescript. Sequencing confirmed that both the clones had identical 3' sequences and both corresponded to the 5' end of MCAK with the 710-bp fragment extending further in the 5' direction beyond the putative consensus start codon for translation.

Production of Antisera to MCAK

MCAK fusion protein was ethanol precipitated and emulsified in Freund's complete adjuvant. Subsequent boosts were emulsified in Freund's incomplete adjuvant. Two mice were injected intraperitoneally with 200 μg of fusion protein per boost. Intraorbital bleeds were taken every other week, and the resulting sera was affinity purified on a small scale on MCAK fusion protein and eluted with 100 mM glycine, pH 2.5. The affinity-purified mouse polyclonal sera was used successfully for immunofluorescence and immunoblots. 1 wk after one of the boosts, the spleen from one of the mice was fused for the production of monoclonal antisera, and one positive clone was obtained that was specific for the MCAK region of the fusion protein. Rabbit polyclonal sera against ethanol-precipitated MCAK fusion protein was produced (Berkeley Antibody Co., Richmond, CA) and affinity purified in a two-step procedure as follows: the sera was centrifuged and run over a maltose-binding protein column to remove antibodies against the maltose-binding protein part of the fusion protein. The flow-through was then run over a column of MCAK-MBP fusion protein. The column was eluted with 100 mM glycine, pH 2.5, and neutralized.

Microtubule Pelleting Assay

20 subconfluent 150-cm^2 plates of CHO cells were rinsed with PBS$^+$ EDTA, and the cells were trypsinized and collected. The cells were rinsed in full-strength fetal calf serum to neutralize the trypsin and were then rinsed in PBS. The 2.0-ml pellet was resuspended in BRB80 buffer (80 mM Pipes, pH 7.0, 1 mM MgCl$_2$, 1 mM EGTA, PMSF, aprotinin, leupeptin) homogenized with a 7-ml Potter-Elvehjem tissue grinder and centrifuged 1 h at 4°C at 100,000 g. Glucose (20 mM) and 20 U/ml yeast hexokinase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added, and the supernatant was incubated for 5 min at room temperature. Taxol (20 μM) and MgGTP (1 mM) were added to the supernatant and incubated for 5 min at room temperature. The supernatant was centrifuged over a 30% glycerol cushion in BRB80, taxol and GTP in a rotor (TLA100.3; Beckman Instruments, Inc., Fullerton, CA) for 20 min at 80,000 rpm in a tabletop ultracentrifuge (Beckman Instruments). The pellet was broken up with a 26-gauge needle in BRB80, taxol and GTP, and was respun. The pellet was resuspended in BRB80, taxol, and GTP plus 5 mM MgATP and 0.25 M potassium acetate. The ATP-extracted microtubules were repelleted through a cushion as before.

Isolation of Interphase Nuclei

Four just subconfluent 150-cm^2 plates of CHO cells were washed with PBS to remove mitotic cells and then the interphase cells were removed from the plates using PBS + 5 mM EDTA. The cells were pelleted and then resuspended for 5 min in 20°C TE (5 mM Tris-HCl, pH 8.0, 1 mM EDTA + PMSF) to swell the cells. Cells were pelleted by centrifuging for 5 min at 900 g. The cell pellet, which was ~1.0–1.5 ml in volume, was resuspended in 2.0 ml ice-cold lysis buffer (TE, pH 8.0, PMSF, aprotinin, leupeptin) and was homogenized for 10 strokes with a 7-ml Dounce homogenizer (Wheaton Instruments, Mays Landing, NJ). Microscopic visual inspection showed full lysis under these conditions and no cytoplasmic debris clinging to the nuclei. The lysis step must be kept free of polyamines and divalent cations, or flocculent cytoplasmic debris will pellet with the

nuclei. No detergent was used because this compromised the integrity of the nuclei. The lysate was layered over a 5-ml cushion of 30% sucrose in lysis buffer + 1 mM spermine and 0.5 mM spermidine (Sigma Immunochemicals, St. Louis, MO) to protect the nuclei during centrifugation. The lysate was centrifuged for 10 min at 5,000 g. The supernatant above the cushion was saved as the cytoplasmic fraction. The nuclear pellet was resuspended in a volume of TE+polyamines equal to that of the cytoplasmic fraction. Fractions were frozen in aliquots in liquid N_2. Equivalent volumes of the nuclear and cytoplasmic fractions were boiled and run on a 10% SDS-PAGE gel and blotted onto 0.2 μm nitrocellulose. Fractions were probed with rabbit anti-MCAK and antitubulin to determine the extent (if any) of cytoplasmic contamination. The extent (if any) of histone (nuclear) contamination was assayed by comparing the nuclear and cytoplasmic fraction on a Coomassie blue–stained 13% SDS-PAGE gel.

Immunocytochemistry and Immunoblots

CHO cells were grown in MEMα with 10% supplemented calf serum, L-glutamine, and pen-strep. Mitotic CHO cells for gels and immunoblots were obtained by mitotic shake-off. All cells were fixed in 4% formaldehyde (Ted Pella Inc., Redding, CA) for 20 min at room temperature, washed, and labeled with either affinity-purified mouse polyclonal sera (straight) or rabbit polyclonal sera (affinity purified) at 1:100, or mouse monoclonal sera (10 μg/ml) for 1 h at room temperature. Donkey anti–mouse secondary antibodies conjugated to fluorescein or Texas red were purchased from Jackson ImmunoResearch Labs, Inc., West Grove, PA). The CHO chromosomes were isolated and prepared for immunocytochemistry as described in Wordeman et al. (1991). Antitubulin label was done with a 1:1 mixture of DMlα and DMlβ, two monoclonals against α- and β-tubulin, respectively. The monoclonal against dynein (mAb 70.1) has been described previously (Steuer et al., 1990; Wordeman et al., 1991). The polyclonal against CENP-B was the kind gift of Bill Earnshaw (Dept. of Cell Biology and Anatomy, Johns Hopkins University, Baltimore, MD). SDS-polyacrylamide gels (8%; Laemmli, 1970) were blotted onto nitrocellulose (Towbin et al., 1979), blocked with 5% Blotto (Carnation; Nestle Food Co., Glendale, CA), and probed with either affinity-purified mouse sera (1:2), the mouse anti-MCAK mAb (5 μg/ml), or affinity-purified rabbit sera (1:500) for 1 h at room temperature. Secondary antibodies conjugated to alkaline phosphatase were purchased from Promega Corp. (Madison, WI). Photomicrographs were photographed on a Photoscope III (Carl Zeiss, Inc., Thornwood, NY) and also on a Nikon FX-A photomicroscope (FX-A; Nikon Inc., Melville, NY) using Kodak Technical Pan Film 2415.

Northern Analysis

Total RNA was produced from CHO cells by an improvement of the method of Chomczynski and Sacchi, 1987 (Meltzer et al., 1990). RNA was run on a 1% agarose gel in the presence of 6.5% formaldehyde and transferred to Hybond N$^+$ (Amersham). The blot was hybridized overnight with 10^8 cpm of two combined random primed (GIBCO BRL) fragments from MCAK (818 bp EcoRI and 1,035 bp XhoI) at 42°C in 50% formamide, 10% dextran sulfate, 1% SDS, 1 mg single-strand DNA in 50 mM Tris-Cl, pH 7.5. After hybridization, the filter was washed 30 min in 2× SSC, 1% SDS at 60°C, followed by 30 min in 0.1× SSC, 0.1% SDS at room temperature. The filter was exposed overnight to Kodak XAR-5 film.

Results

Identification and Cloning of MCAK

Affinity-purified rabbit antisera raised against two conserved regions of the kinesin motor domain were used to screen a λ-ZAP expression library made from CHO cell cDNA. The two antisera, termed anti-LAGSE and anti-HYPIR, respectively, recognized a large number of clones from this library. All clones that cross-reacted to a greater or lesser extent with both antisera were subsequently identified as kinesins by sequence analysis. Novel kinesins, identified via sequence, were subcloned into the pMALc-RI vector (New England Biolabs) which was transformed into three reading frames as described in Materials and Methods. Mouse polyclonal antibodies were produced against several of the clones and
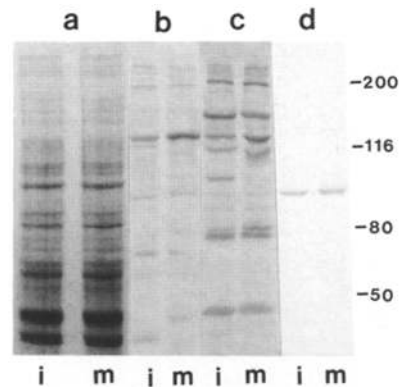

Figure 1. Immunoblot analysis of mitotic and interphase CHO cells. Coomassie gel of interphase (i) and mitotic (m) CHO cells (a). Corresponding immunoblots probed with anti-HYPIR (b), anti-LAGSE (c), and anti-MCAK monoclonal (d). Molecular mass markers in kilodaltons are shown at the right.

affinity-purified against fusion protein. Affinity-purified antisera were screened immunofluorescently on mitotic CHO cells and, in this way, novel spindle-associated kinesins were identified. The immunoblot in Fig. 1 shows repeated lanes of interphase and mitotic CHO cells. Lane pairs (Fig. 1, b and c) show the spectrum of putative kinesin-related proteins as recognized by anti-LAGSE and anti-HYPIR, respectively.

One clone, hereafter termed MCAK, was identified in this manner. Lane pair d in Fig. 1 shows interphase and mitotic CHO cells probed with the mouse polyclonal antiserum against MCAK. Anti-MCAK recognizes a single band of 90 kD. The antiserum shows no cross-reactivity to any of the other putative kinesin-related proteins recognized by the antipeptide sera. In addition to the affinity-purified mouse polyclonal antiserum originally used to identify and characterize MCAK, we produced affinity-purified rabbit antiserum and a mouse monoclonal against MCAK. All three antibodies: mouse polyclonal, rabbit polyclonal, and mouse monoclonal gave similar results by immunofluorescence and on immunoblots.

Fig. 2 shows the immunofluorescent localization in CHO cells at different stages of the cell cycle. Panels a–c show a field of cells that have been triple labeled with affinity-purified anti-MCAK, antitubulin, and Hoescht stain, respectively. In this field, all the cells are in interphase, except one in the lower left corner, which is in prophase (this is clearly seen in the Hoescht DNA label in Fig. 2 c). Varying levels of diffuse label can be seen in the cytoplasm and nuclei of the interphase cells. This is typical. Attempts were made to determine whether more brightly labeled nuclei corresponded to those cells that were farther along in the cell cycle. No statistically significant correlation between MCAK label intensity and position in the cell cycle were observed. MCAK labeled centromeres in an early metaphase CHO cell is shown in Fig. 2 d, accompanied by DNA label (Fig. 2 e). The mitotic spindle is oriented longitudinally in these two micrographs. The MCAK protein appears to stretch between the two sister kinetochores. Fig. 2, f and g, shows two late telophase sister cells in which the remnant of the mitotic spindle is oriented horizontally (the microtubules are not labeled). It can be clearly seen in Fig. 2 f that the MCAK label is still visible on the centromeres of the recently divided nuclei. This labeling pattern does not persist for long. As

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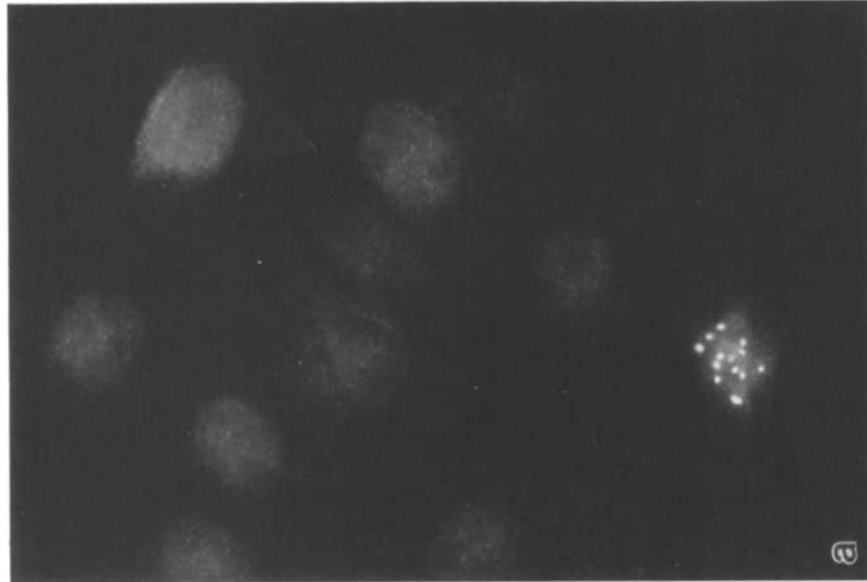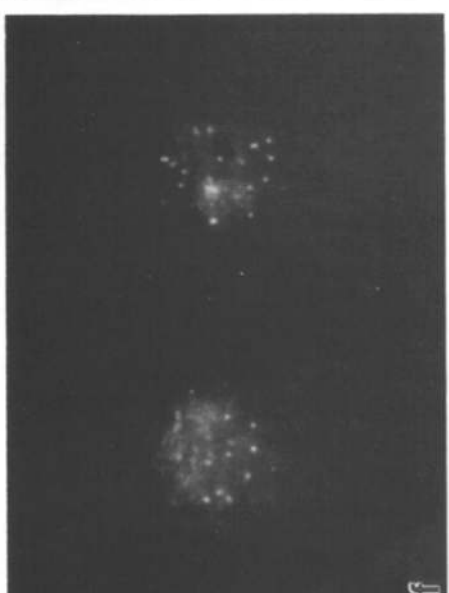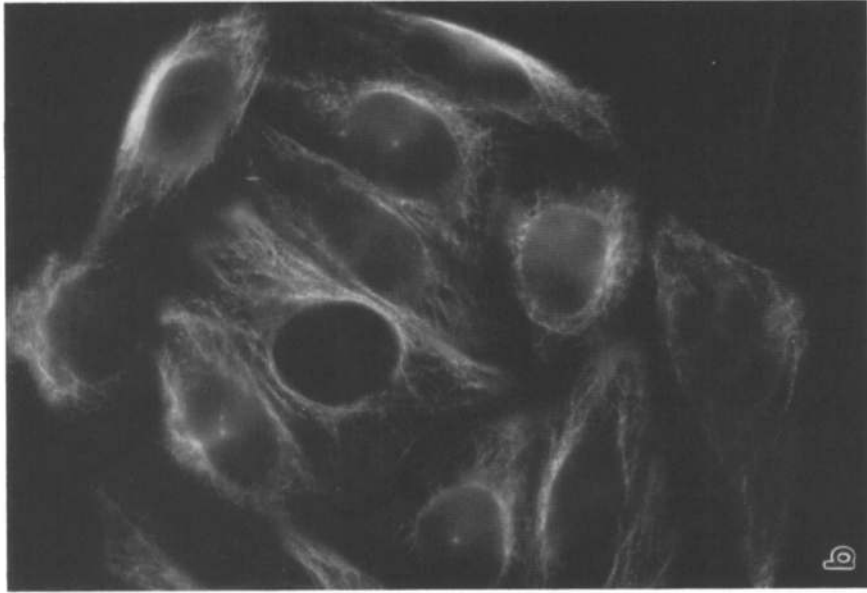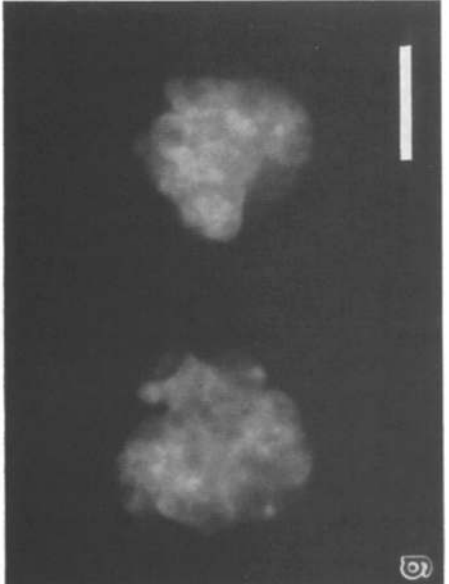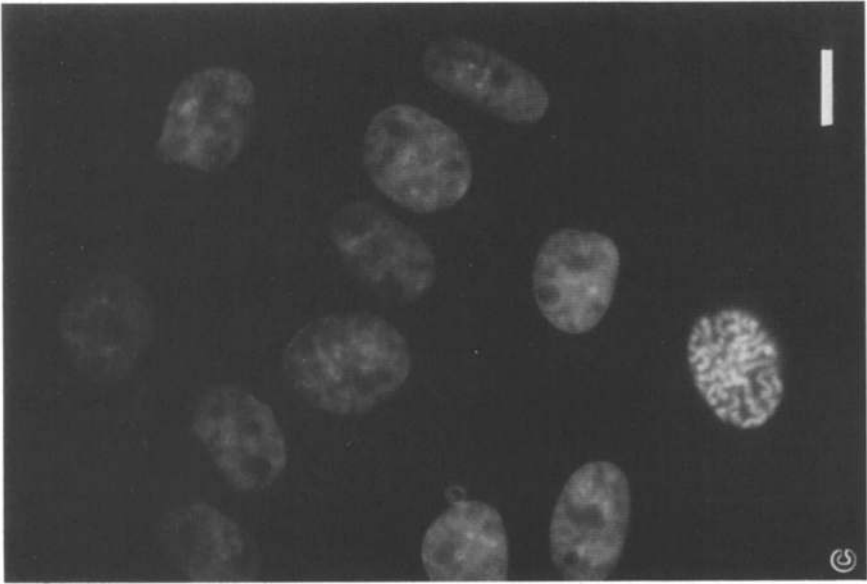
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                                                    M  E  S  L     4
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       P  A  R  L  F  P  G  L  S  I  K  I  Q  R  S  N  G  L  I  H   24
 121  CAGTGCCAACATAAGCACAGTGGAATGGATAGAAGGAGGTAACACGAAAGGCAAAGAGAT
       S  A  N  I  S  T  V  E  W  I  E  G  G  N  T  K  G  K  E  I   44
 181  TGATTTTGATGATGTGGCTGCAATAAACCCAGAACTTCTACAGCTCTTCCCTTACACCCA
       D  F  D  D  V  A  A  I  N  P  E  L  L  Q  L  F  P  Y  T  Q   64
 241  AAGACAATCTGCCACTGCAGGAAAATGTAACAGTTCCGAAGCAAAAACGCATATCAGTCA
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 301  ACTCCAAAATTCCAGCTCCAAAAGAAGGTCTTCGAAGCGTTCCACTCGCATGTCCACTGT
       L  Q  N  S  S  S  K  R  R  S  S  K  R  S  T  R  M  S  T  V   104
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 421  AACTCTCGAAAGCAGTTTTCGGTTGGCCACTGGCCTCCCTAGGCCCTCCTGTCCTGCAAT
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1021  AAAAGCAACCTGCTTTGGCTATGGGCAGACGGGCAGTGGGAAGACTCACACAATGGGTGG
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       D  C  L  V  N  L  R  I  H  L  K  G  I  L  C  F  L  L  K  S   364
1141  TCAGCCTCGCTACCGGAACTTAAACTTGGAAGTTTATGTGACATTCTTTGAGATCTACAA
       Q  P  R  Y  R  N  L  N  L  E  V  Y  V  T  F  F  E  I  Y  N   384
1201  TGGGAAGGTGTTTGATCTGCTCAACAAGAAGGCCAAGCTACGTGTACTAGAAGACAGCAA
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1381  TTCTTCCCGCTCCCATGCCTGCTTCCAGATTCTTCTTCGAGCCAAAGGGAGATTGCATGG
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1441  CAAATTCTCCTTGGTGGATCTGGCAGGGAATGAACGAGGGGCTGACACTTCTAGTGCTGA
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1501  CCGCCAGACTCGCATGGAGGGTGCAGAGATTAACAAGTCTCTCCTAGCACTGAAGGAGTG
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1561  CATCAGGGCGCTGGGACAGAACAAGGCTCACACCCCATTCCGTGAGAGCAAGCTGACTCA
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1801  GATGGAAGCCAGCTCTAATGGACCTCGCTGGCAGTCAATTTCAAGGAAGAAGAGGAGCT
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1861  GTCTTCCCAGATGTCCAGCTTTAATGAAGCCATGAGTCAGATCAGGGAGCTGGAAGAGAG
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1921  GGCCATGGAGGAGCTCAGGGAGATCATACAGCAAGGACCAGGCTGGCTTGAGCTCTCTGA
       A  M  E  E  L  R  E  I  I  Q  Q  G  P  G  W  L  E  L  S  E   644
1981  GATGACTGATCAGCCAGACTATGACTTAGAGACCTTTGTGAACAAAGCAGAATCTGCCCT
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2041  GACTCAGCAGACTAAGCACTTCTCAGCCCTGCGAGAAGTCATCAAGGCCCTGCGTGTGGC
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2101  CATGCAACTGGAAGAACAGGCTACCAACACAGATGAACAGCAAGAAACGGCACCAATGAT
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2161  GACTTCAATAAAGATCTGTTTTATGTCACACCAGCCTCTTCCCCTATCCAGCAAACTTTGG
       T  S  I  K  I  C  L  C  H  T  S  L  F  P  Y  P  A  N  F  G   724
2221  GCACCTTCTGGCTTTGGTTAGGGACTGAGTTGGATGGGTTTTGGTAAATGCCAGGTAAGG
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2281  GGCATCAGGCCCAGGAAGACTCGGAGAGAGAATTGCTGTTCTCTTGCTTAGTTTGGAGCA
2341  GGAGGAAGGAGCTTTTCATGACTGCTTCCCCTTTTCCTCCCTATCAGAGGGGGTGCTGTT
2401  TGGGTAGTCTCCCTGCGGCAGCACCCTGCCCCTCTGCAACAGCTGCTGATAGACAACCTG
2461  CAAGGTCCAGGCTCTGGGAGAGAAATTGGTCCTCGTCCAGCTTCTACTGCCTTTGGTCTG
2521  TGGTATCACCCTTGCTCTGAGCACTCTTGTGTCAGAAAAAAAAAAGTTTTTGAGACCTCT
2581  GTTTTAGTTTTAGAAATCCAAGATTTCCATTTCCTGCTGTATGTATTTTTATGTTGTACC
2641  TAACAATAAAGAAAGAAAAAGTCCAAAAAAAAAAAAAAAAAAAAAAA  2687
```

Figure 3. Nucleotide and predicted amino acid sequence of MCAK. The nucleotide residues are numbered on the left and the amino acid residues are numbered on the right. The nucleotide sequence corresponding to the eukaryotic consensus start codon is underlined. The peptide sequences recognized by the affinity-purified antipeptide sera are also underlined.

Figure 2. Anti-MCAK immunofluorescence of CHO cells. (*a–c*) A field of interphase CHO cells is shown with one prophase cell visible in the lower left, anti-MCAK- (*a*), antitubulin- (*b*), and Hoechst-stained DNA (*c*). Interphase cells exhibited a small amount of diffuse label in the nucleus and cytoplasm. Brightly labeled kinetochores became visible during prophase. Bar, 20 μm. Early metaphase CHO cell labeled with anti-MCAK (*d*) and Hoechst (*e*). Late telophase CHO cell, anti-MCAK (*f*), and Hoechst (*g*). Bar, 8 μm.

soon as the cell has completed telophase, the centromere label is no longer evident.

MCAK is a Kinesin-related Protein

Fig. 3 shows the nucleotide and amino acid sequence of MCAK. Two conserved regions diagnostic for the kinesin motor domain and recognized by the antipeptide sera are shown underlined. Also, the nucleotide sequence that corresponds to the eukaryotic consensus sequence for translation initiation is shown underlined (Kozak, 1986). The open reading frame is sufficient to code for a 90-kD protein.

Using immunoblots and anti-MCAK antibodies, we have determined that MCAK binds to bovine microtubules polymerized in ATP-depleted high speed supernatants of CHO cells. This is shown in Fig. 4. Lanes *A–E* correspond to a Coomassie-stained gel of a typical microtubule pelleting assay from CHO cells. The same lanes were blotted onto nitrocellulose and probed with rabbit anti-MCAK in Fig. 4, *a–e*. MCAK will bind to microtubules in ATP-depleted cell supernatants. MCAK is released from microtubules in the presence of 5 mM ATP and 250 mM KAcetate. Addition of either 5 mM ATP or 250 mM KAcetate alone will not release MCAK from microtubule pellets (data not shown).

Subcellular Distribution of MCAK

The appearance of MCAK in the high speed supernatants used for the microtubule pelleting assay suggested that interphase cells have MCAK in the cytoplasm. However, the immunofluorescence data suggests that there is also MCAK in interphase nuclei. We separated interphase CHO cells into nuclear and cytoplasmic fractions and probed the fractions with rabbit anti-MCAK. Fig. 5 *B* indicates that approximately equal levels of MCAK are found in the cytoplasm and the nucleus. Lane pair *A* and *D* show, respectively, 10% and 13% Coomassie-stained SDS-PAGE gels of the fractions. The 13% gel (lane *D*) shows no histone contamination in the cytoplasmic fraction. Immunoblot C is identical to B, except that it is probed with antitubulin as a marker for cytoplasmic proteins. No antitubulin label is seen in the nuclear fraction, indicating that it is free of cytoplasmic contamination. Hence, during interphase, MCAK protein can be found both in the nucleus and in the cytoplasm.
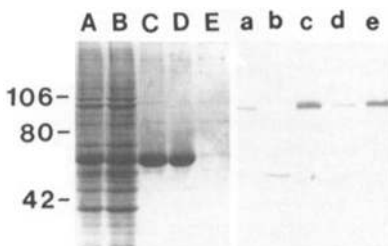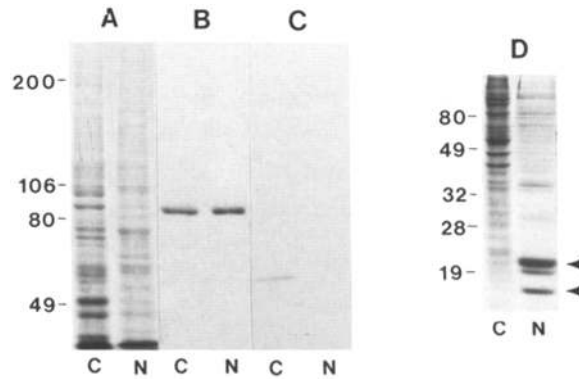

Figure 5. MCAK is found in the cytoplasm and in the nucleus during interphase. (*A–C*) Successive lanes of CHO cytoplasm (*C*) and nuclei (*N*) run on a 10% SDS-PAGE gel and Coomassie stained (*A*), electrophoresed onto nitrocellulose, and probed with anti-MCAK (*B*) or antitubulin (*C*). Molecular mass standards in kilodaltons are shown to the left. (*D*) 13% SDS-PAGE gel of cytoplasmic (*C*) and nuclear (*N*) fractions showing histones (*arrowheads*). Molecular masses are on the left.

Northern Analysis of MCAK

We isolated total RNA from CHO cells to determine the size and number of the MCAK message(s). A 2-kb fragment from the MCAK clone used to produce the antiserum was labeled and used to probe a Northern blot of 8 μg total CHO cell RNA. Fig. 6 shows that a single message of 3.4 kb is recognized by the probe. This is in close agreement with the 3.5-kb reported size for the major transcript of kif2 in adult mouse brain. Poly-A+ messenger RNA was isolated from total RNA using oligo-dT cellulose and the same size single transcript was found (not shown). This transcript is of sufficient size to include the full 2,738-bp clone shown in Fig. 2. We believe that in these cells, only one MCAK transcript is being produced.

MCAK Binds to the Centromere Region of Mitotic Chromosomes

In Fig. 7, we compare the labeling pattern of MCAK with CENP-B, dynein, and tubulin on three different isolated mitotic CHO cell chromosomes. CENP-B is a DNA-binding protein that is presumably involved in organizing the struc-
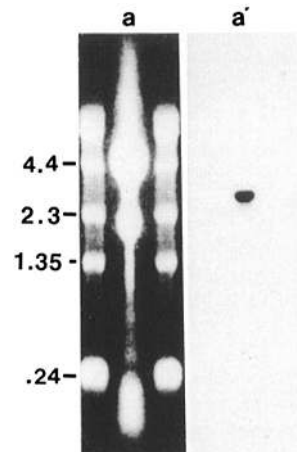

Figure 4. MCAK binds and releases microtubules in an ATP-dependent manner. (*A–E*) 10% Coomassie gel and corresponding anti-MCAK immunoblot (*a–e*) of a microtubule pelleting assay from CHO cells. (*A* and *a*) High speed supernatant of CHO cells. (*B* and *b*) Microtubule-depleted high speed supernatant. A proteolytic fragment in (*b*) results from the 20°C incubation. (*C* and *c*) Microtubule pellet. (*D* and *d*) ATP-extracted microtubule pellet. (*E* and *e*) ATP extract. Molecular mass standards in kilodaltons are shown on the left.

Figure 6. Northern immunoblot of total CHO cell RNA probed with cloned MCAK fragment. (*a*) Shows a formaldehyde gel of total CHO cell RNA and RNA markers labeled in kilobases. (*b*) Shows the Northern blot from the same gel probed with a labeled 2-kb fragment of the MCAK clone.

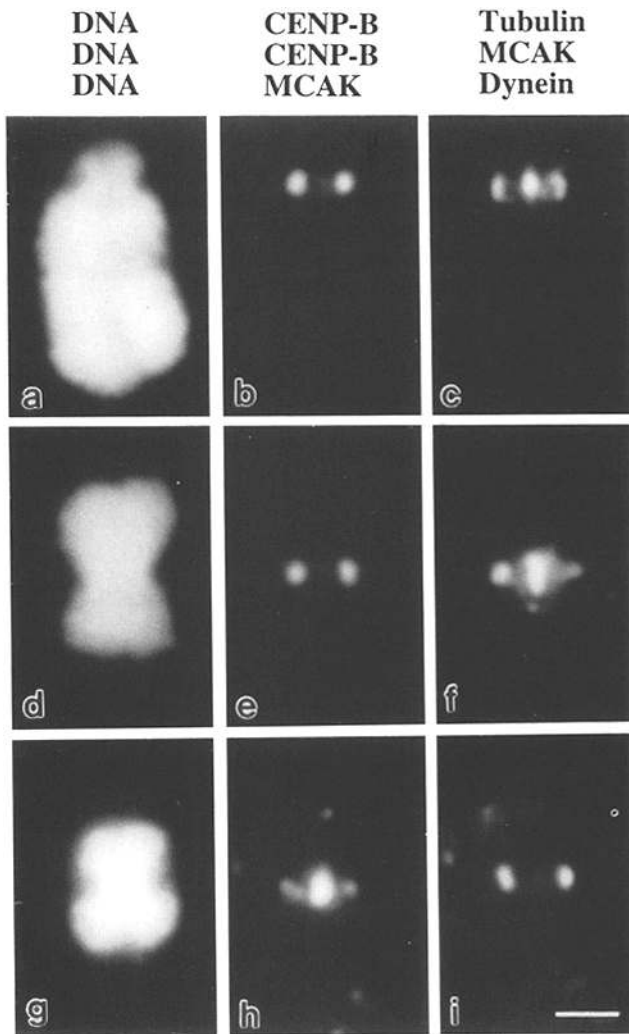| DNA	CENP-B	Tubulin
DNA	CENP-B	MCAK
DNA	MCAK	Dynein

Figure 7. Anti-MCAK localization in isolated mitotic CHO chromosomes resembles that of bound tubulin. Isolated mitotic CHO chromosomes were incubated with tubulin subunits for 5 min at room temperature, fixed, and labeled for indirect immunofluorescence. (*a–c*) A mitotic CHO chromosome double labeled with CENP-B (*b*) and antitubulin (*c*). (*d–c*) Another chromosome labeled with CENP-B (*e*) and anti-MCAK (*f*). A comparison of anti-MCAK label (*h*) and antidynein (*i*). Bar, 1 μm.

ture of the centromere (Cooke et al., 1990; Bernat et al., 1991), and we use it in this study as a marker for the fundamental structural unit of the centromere. It has been previously shown that isolated mitotic CHO chromosomes bind monomeric tubulin at the outer corona region of the kinetochore (Mitchison and Kirschner, 1985) distal to the kinetochore plate. In Fig. 7, *a–c*, we compare the localization of bound tubulin (*c*) with that of CENP-B (*b*). We often see tubulin bound both on the corona of the two sister kinetochores and between the kinetochores in the centromere region (Fig. 7 *c*) after incubation in tubulin. This pattern of tubulin binding becomes even more interesting when the immunofluorescence pattern of anti-MCAK and anti-CENP-B are compared (Fig. 7, *d–f*). A chromosome double-labeled with anti–CENP-B and anti-MCAK shows MCAK

label to be profuse in the region between the sister kinetochores and extending distally in either direction (Fig. 7 *f*). Although this chromosome has been preincubated with tubulin, the MCAK pattern is identical in chromosomes that have not been pretreated in any way (not shown). We find the central region of MCAK label to be roughly coincident with the central blob of antitubulin label. We were surprised to see a microtubule-dependent motor such as MCAK distributed throughout the centromere region instead of on the outer face of the kinetochore, where microtubule interactions are most likely to take place. This localization pattern is in contrast to the two other kinetochore-associated microtubule-dependent motors: antidynein (Fig. 7 *i*) and anti–CENP-E (not shown), which labels the outer face and corona region of the kinetochore (Pfarr et al., 1990; Steuer et al., 1990; Wordeman et al., 1991; Yen et al., 1992). The contrast between the centromeric distribution of MCAK and that of cytoplasmic dynein is shown in the double-labeled chromosome in Fig. 7, *g–i*. Unlike the discrete labeling of the outer kinetochore region with antidynein (Fig. 7 *i*) and CENP-E (Yen et al., 1991, 1992), anti-MCAK label of the centromere region of CHO cells appears irregular (Figs. 1 and 7, *f* and *h*).

Predicted Features of MCAK Peptide Sequence

One interesting feature of the MCAK amino acid sequence is the location of the motor domain. The diagram in Fig. 8 *A* illustrates that instead of being displaced toward the amino-terminal end of the polypeptide, as is the case for most identified kinesins, or at the carboxy-terminal end of the protein, as in ncd and kar3, the motor domain is in the middle of the peptide. The location of the ATP-binding region is indicated. This is unusual and has also been described for kif2, a kinesin-related protein originally isolated from mouse brain and subsequently found to be represented in many other tissues. (Aizawa et al., 1992). The secondary structure prediction using three different algorithms is shown in Fig. 8 *B*. There are extended regions of predicted helix domain in the region COOH-terminal to the more globular motor domain. The length and alignment of Fig. 8 *B* corresponds to the diagram in Fig. 8 *A*. Within both the NH₂-terminal and COOH-terminal nonmotor domains are, respectively, two regions of predicted coiled-coil (Lupas et al., 1991) that might be involved in interactions with DNA, light chains, or heavy chains. Coiled-coil regions of probability 98% stretch from amino acids 178 to 208 in the amino-terminal region of the protein, and from amino acids 597 to 632 in the carboxy-terminal tail of the protein. These regions are diagrammed in Fig. 8 *A*, and they line up with helix predictions in Fig. 8 *B*. Because a large proportion of MCAK is found in the interphase nucleus, we looked for putative nuclear localization sequences (Chelsky et al., 1989). A region that fits the consensus squence K-R/K-X-R/K is shown in Fig. 8 *C*. It resides within a predicted helix-rich region of the motor domain.

Because a centrally located motor domain has been described for kif2, we performed a peptide matrix comparison of MCAK and kif2. The plot is shown in Fig. 9. The considerable identity between the MCAK and kif2 sequence suggest that they are members of the same class of kinesin-related proteins (Goldstein, 1993*a*).
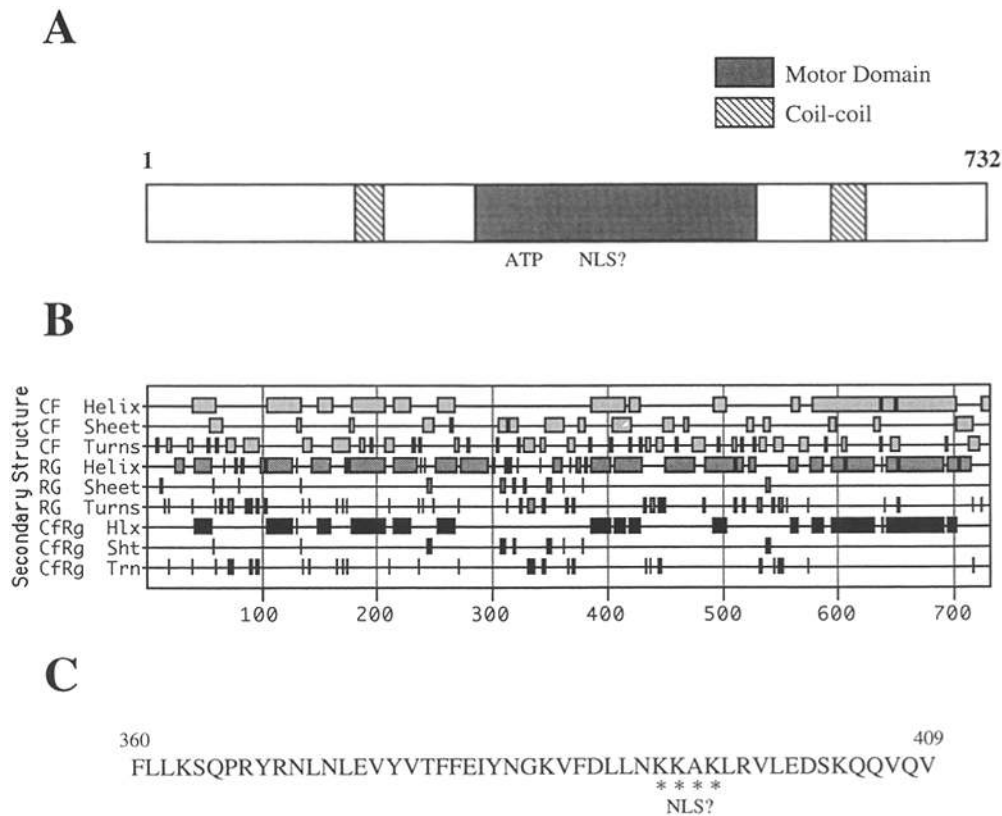
A

B

C

360   409
FLLKSQPRYRNLNLEVYVTFFEIYNGKVFDLLNKKAKLRVLEDSKQQVQV
                              * * * *
                               NLS?

Figure 8. MCAK secondary structure predictions. (*A*) Diagram of MCAK peptide sequence showing the location of the motor domain, the ATP-binding site, a putative nuclear localization sequence, and predicted regions of coil-coil domains. (*B*) Secondary structure predictions of MCAK using three different algorithms (Macvector 4.1.4 - Kodak SIS). (*C*) Detail of peptide sequence surrounding the putative nuclear localization consensus sequence.

Protein(s) Antigenically Related to MCAK Have Been Found in All Species Tested by Western Immunoblot

The monoclonal antibody produced against MCAK cross-reacts both immunofluorescently and on immunoblots against all organisms that we have tested. Fig. 10 shows an immunoblot of cell extracts from a variety of organisms probed with the anti-MCAK monoclonal. A 90-kD band is detected in all vertebrates (Fig. 10, *a* and *b*). At least one and sometimes two bands are detected in all other organisms, including *S. cerevisiae* (Fig. 10 *f*). The molecular masses of the antigenically related bands begin to deviate from 90 kD in the invertebrates (Fig. 10, *c–f*). The low molecular mass faint bands in Fig. 10, *a*, *d*, and *f*, are the result of proteolysis and

Window Size = 8
Min. % Score = 6 0
Hash Value = 2
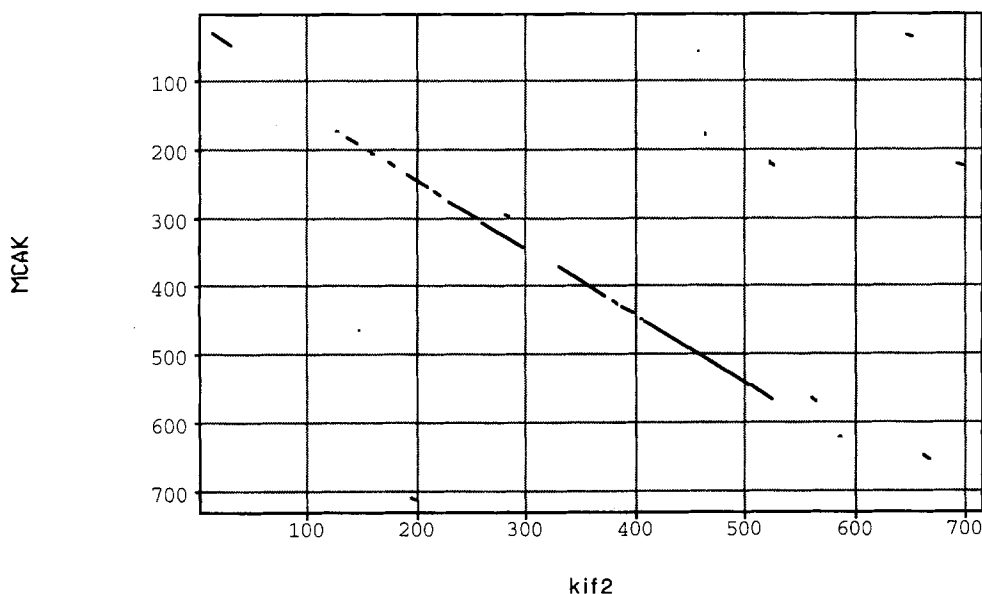
Scoring Matrix: pam250 matrix

Figure 9. Matrix plot comparison of MCAK and kif2. PAM250 scoring matrix (Pearson, 1990) of MCAK and kif2.
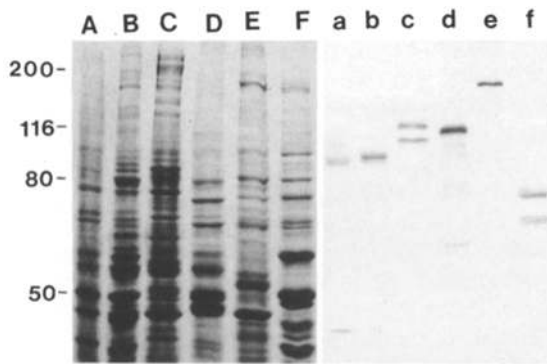
102

Figure 10. Anti-MCAK monoclonal recognizes antigenically related peptides in other organisms. A 10% SDS-polyacrylamide Coomassie gel is shown in *a-g*. The lanes are 0.1-mg protein samples of the following: (*a*) interphase CHO cells, (*b*) *Xenopus laevis* egg extract, (*c*) *Strongylocentrotus purpuratus* egg extract, (*d*) *D. melanogaster* egg extract, (*e*) *Dictyostelium discoideum* amoebae, and (*f*) *S. cerevisiae* extract. The corresponding immunoblot probed with the anti-MCAK monoclonal is shown in *a'-g'*. Molecular mass markers in kilodaltons are shown on left.

are reduced or eliminated when proteolytic inhibitors are added to the cells during homogenization (data not shown). MCAK seems to be quite sensitive to proteolysis and is rapidly degraded when proteolytic inhibitors are left out of the preparation.

Discussion

We have cloned a kinesin-related protein from a CHO cell cDNA library using affinity-purified peptide antibodies raised against conserved regions of the kinesin motor domain (Sawin et al., 1992). We chose to focus more closely on one clone in particular because serum raised against expressed fusion protein from this clone labeled the centromere region of mitotic chromosomes. For this reason, we call this protein MCAK, for mitotic centromere-associated kinesin. The full-length MCAK clone contains an open reading frame capable of coding for a 95-kD protein, which is in close agreement with the 90-kD species that is detected by anti-MCAK antibodies in CHO cells. An in-frame methionine was chosen as the start codon because it was flanked by the eukaryotic consensus sequence for initiation of translation, ACCATGG (Kozak, 1986). Sequence analysis shows that MCAK is ~70% similar to kif2, a kinesin-related protein cloned from mouse brain, but also demonstrated to be found in all mouse tissues tested (Aizawa et al., 1992). MCAK appears to be coded for by a single abundant message of 3.4 kb. Antisera produced against MCAK labeled the centromere region of chromosomes before nuclear envelope breakdown and remained detectable at mitotic centromeres until after telophase, at which time the specific labeling of centromeres disappeared. The protein is also found in the centromere region of isolated mitotic chromosomes, and it colocalizes with bound monomeric tubulin.

MCAK is highly homologous to kif2 (40-50% identity, 73% within the motor domain), and is likely to be a member of the same class of kinesin-related proteins. Both kinesins exhibit the unusual property of having the motor domain

centrally located within the peptide. Previously, kinesins have been described as having the motor domain displaced either toward the NH₂-terminal or COOH-terminal end of the protein. Interestingly, this feature seems to predict the directionality of the kinesin, despite the fact that the directionality is determined by factors intrinsic to the motor domain irrespective of its position (Stewart et al., 1993). In all cases in which successful gliding motility assays have been performed, NH₂-term kinesins have moved toward the plus end of microtubules, and all COOH-term kinesins have moved toward the minus end of microtubules (Vale et al., 1985*b*; McDonald et al., 1990). Therefore, the direction of a centrally located motor like kif2 or MCAK is of great interest. However, preliminary motility assays with bacterially expressed MCAK have been disappointing. The protein will bind to microtubules in the absence of ATP (controls with inactive MCAK will not bind microtubules under any conditions). The addition of ATP and 0.2 M potassium acetate will cause MCAK to release the microtubules without any sign of gliding motility in either direction. Further, work needs to be done with this protein to resolve this tantalizing issue.

Predictions of secondary structure (Chou-Fasman) for both kif2 (Aizawa et al., 1992) and MCAK show an NH₂-terminal globular domain, a globular motor domain, and a COOH-terminal tail that has long stretches of predicted α-helix. For this reason, it has been suggested that the protein may exist as a dimer in solution, with dimerization being mediated by the COOH-terminal helical region (Goldstein, 1993*a*). Furthermore, a program that predicts coil-coil domains (Lupas et al., 1991) has identified one region of coil-coil stretching for ~30 amino acids on either side of the centrally located motor domain. These regions may mediate the binding of light chains, DNA, or other MCAK heavy chains.

There is no evidence to suggest that kif2 is a motor protein that has any role in mitosis. However, another kinesin-related protein has been isolated from the diatom *Cylindrotheca fusiformis*. This 95-kD protein is associated with the mitotic spindle, and it cross-reacts with the anti-MCAK monoclonal. It is not known if the protein is associated with kinetochores in this organism. The diatom protein shares a large amount of identity with MCAK and has a centrally located motor domain like MCAK and kif2 (Foss, M., H. Wein and W. Z. Cande, personal communication).

During interphase, diffuse nuclear staining of variable intensity is observed with anti-MCAK antisera. We looked for a nuclear localization concensus sequence within the peptide sequence of MCAK. We found one sequence within the motor domain that fits a four-residue consensus sequence (K-K/R-X-K/R) found to be sufficient to induce nuclear targeting with synthetic peptides (Chelsky et al., 1989). A search for similar sequences in other known kinesins revealed inconsistent results. Many kinesins contained some variation of the above consensus sequence, and only one of the several that we tested (bimC) contained the K-K-R-K sequence (Kalderon et al., 1984). We are presently preparing to perform site-directed mutagenesis with epitope-tagged MCAK in CHO cells to test whether the K-K-A-K sequence in the motor domain of MCAK is a functioning nuclear localization sequence.

We are presently screening the library for any other possible MCAK-related clones. During a subsequent screen of the CHO-K1 library, we determined that ~25% of our positive

plaques cross-hybridize with MCAK. It may be that, for whatever reason, MCAK is highly represented in the CHO library. Or it may be that there are several closely related but distinct MCAK transcripts. Or, indeed, both these possible scenarios may be true. The recent identification of a hetero-trimeric kinesin-related protein (Cole et al., 1993) raises the possibility that, in addition to homodimers, different kine-sin-related proteins may form heterodimers or -trimers in the cell. The formation of heterodimeric kinesins may account for the large number and variety of kinesin-related proteins expressed in the cell. To design experiments to test the function of MCAK within the cell, it will be necessary to examine the native form of the molecule and identify any associated proteins. Both the mouse monoclonal anti-MCAK and the affinity-purified rabbit anti-MCAK bind native protein in immunoprecipitations. We hope to isolate the native form of MCAK to further explore this issue.

All antisera produced against MCAK labels the centromere region of mitotic chromosomes. Bright label appears in prophase nuclei before nuclear envelope breakdown and also before the appearance of another kinetochore-associated kinesin, CENP-E (data not shown). Both MCAK and CENP-E are kinesin-related proteins that appear on kinetochores at prophase. However, they are distinctly different proteins. CENP-E is 312-kD protein, with an NH₂-terminal motor domain, that accumulates in G2, associates with kinetochores at prophase, and relocates to the spindle midzone at metaphase. MCAK is a 90-kD protein, with a centrally located motor domain, that is present throughout the cell cycle, associates with centromeres at early prophase, and remains associated with the centromere until after telophase. CENP-E is a rare protein, whereas MCAK may be abundant. Furthermore, these two proteins have a distinctly different location within the centromere region of the mitotic chromosome that is discernible at the level of the light microscope. Therefore, it is likely that these two kinetochore-associated kinesins have different functions during mitosis. CENP-E is distally located relative to MCAK, closer to the outer face and the corona of the kinetochore. Reider and Alexander (1990) have shown that the corona is a site of rapid microtubule gliding activity in mitotic chromosomes. The outer surface of the kinetochore may be the region that mediates much of the plus end– and minus end–directed gliding activity along microtubules. A preliminary suggestion, based solely on localization data, might be that MCAK uses its ATP-dependent ability to bind to microtubules to anchor the kinetochore to the microtubules or to modulate the dynamics of microtubule ends inserted at the kinetochore. CENP-E, on the other hand, may use plus end–directed motor activity to move mitotic chromosomes to the spindle midzone at prometaphase and anchor them there at metaphase.

MCAK's peculiar location, internal to where most of the motor activity is presumed to occur, may reflect a fundamentally different role in chromosome movement than would be expected from a microtubule motor protein. Kinetochores can attach to the ends of depolymerizing microtubules and move in a polewards direction in vitro (Coue et al., 1991). However, in the mitotic spindle, many microtubules are embedded in the kinetochore plate and the chromosomes undergo many reversals in direction (Skibbens et al., 1993). In this situation, the tens of microtubules that are attached to

any one kinetochore must undergo coordinated polymerization and depolymerization. During prometaphase, a pair of sister chromosomes moving in one direction involves the coordinate polymerization of microtubules at one kinetochore and depolymerization at the other kinetochore. A motor protein that is more deeply embedded in the kinetochore may be used to anchor the centromere firmly to the dynamic microtubule ends, or may be involved in modulating microtubule dynamics. Immunoelectron microscopy must be performed to determine if MCAK labeling extends far enough into the kinetochore plate, from the center of the centromere, to encounter microtubule ends. Another possible model to explain the localization data is that MCAK shuttles tubulin subunits from one side of the centromere to the sister side during rapid prometaphase movements and reversals in direction. It is essential to determine if the MCAK/tubulin colocalization in the centromere reflects MCAK's ability to bind tubulin subunits, and if so, if this ability is functionally relevant.

In conclusion, we have presented preliminary evidence for a kinesin-related protein that localizes to the mitotic centromere. This protein, MCAK, is distinct from the kinesin-related protein CENP-E that is found on mitotic kinetochores. MCAK is a 90-kD protein that has the ATP-binding motor domain in the middle of the peptide sequence instead of near the amino- or carboxy-terminal. It appears on prophase centromeres and remains associated with the centromere throughout mitosis. During interphase, it exhibits faint diffuse staining of nuclei and cytoplasm. MCAK is transcribed as a single 3.4-kb message.

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