Localization of cytoplasmic dynein to mitotic spindles and kinetochores

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WHAT is the origin of the forces generating chromosome and spindle movements in mitosis? Both microtubule dynamics and microtubule-dependent motors have been proposed as the source of these motor forces. Cytoplasmic dynein and kinesin are two soluble proteins that power membranous organelle movements on microtubules. Kinesin directs movement of organelles to the ‘plus’ end of microtubules, and is found at the mitotic spindle in sea urchin embryos, but not in mammalian cells. Cytoplasmic dynein translocates organelles to the ‘minus’ end of microtubules, and is composed of two heavy chains and several light chains. We report here that monoclonal antibodies to two of these subunits and to another polypeptide that associates with dynein localize the protein to the mitotic spindle and to the kinetochores of isolated chromosomes, suggesting that cytoplasmic dynein is important in powering movements of the spindle and chromosomes in dividing cells.

Two monoclonal antibodies (440.1 and 440.4) against the heavy chain of chick brain cytoplasmic dynein react with a single band of relative molecular mass 440,000 (M,440K) in purified dynein preparations and in crude cell supernatants of chick fibroblast cells, as shown by immunoblots (Fig. 1). In addition, cleavage of the heavy chain into two fragments of ~200K by exposure to ultraviolet light in the presence of ATP and vanadate demonstrates that the two antibodies react exclusively with the lower M, fragment (Fig. 2). The monoclonal antibodies against the ~150K dynein-associated polypeptide doublet (150.1) and the triplet of ~70K light chains (70.1) each react specifically with bands of the appropriate M, in immunoblots of cell supernatants of chick embryo fibroblasts (CEF) (Fig. 1). No other bands are seen in immunoblots of whole CEF cell lysates.

In intact mitotic CEF cells, the staining of the spindle with any of the anti-dynein antibodies is clearly visible through diffuse cytoplasmic staining. Detergent extraction of the cells in the presence of a microtubule-stabilizing buffer before fixation preserves spindle staining but markedly reduces cytoplasmic staining (Fig. 3), suggesting that dynein is present on the spindle during mitosis and is not there fortuitously as a result of fixation of whole cells.

Interphase cells show diffuse punctate staining throughout the cytoplasm with all the anti-dynein antibodies, consistent with observations that cytoplasmic dynein is a soluble protein in cytosolic extracts of cells. After detergent extraction, the staining is much fainter but still generally diffuse with punctate regions (Fig. 3). The staining in intact interphase cells does not resemble a microtubule pattern, although in extracted cells the punctate staining areas are occasionally aligned along microtubules, further supporting the view that cytoplasmic dynein is a soluble motor protein in interphase cells in vivo.

Throughout metaphase and anaphase, CEF cells show strong anti-dynein staining near the polar and kinetochore microtubules and the spindle poles (Fig. 3). During prometaphase, staining of the organizing spindle is weak, but there is bright, punctate staining of the chromosomes, suggestive of kinetochore...
staining. Cells in telophase show staining of the spindle poles and frequently the midbody. In immunofluorescence experiments with the other antibodies, we consistently observe an identical staining pattern consisting of the central portion of the mitotic spindle.

To determine if the staining of the spindle was on kinetochores or non-kinetochore microtubules, we extracted CEF cells with 0.5% Triton X-100 at 4°C in the presence of 80 μM CaCl₂ to depolymerize all but the kinetochore microtubules. CEF cells treated in this manner still show intense spindle staining with

FIG. 3 Localization of cytoplasmic dynein to the mitotic spindle in dividing CEF cells extracted with a detergent-containing, microtubule-stabilizing buffer (rows 1–4) or microtubule-destabilizing buffer (row 5) before fixation, also stained for tubulin and chromosomes. Row 1, interphase CEF cell; 2, prometaphase CEF cell; 3, metaphase CEF cell; 4, anaphase CEF cell; and 5, metaphase cell, extracted before fixation under conditions designed to depolymerize all but the kinetochore microtubules. Cells were stained for dynein (a, c, f, i), tubulin (b, d, g, m) and chromosomes (e, h, k, n). Bar, 10 μm.

METHODS. Primary CEFs were prepared from 11-day-old chick embryos and grown in 10-cm dishes. Secondary CEFs were plated on coverslips 16–24 h before use. For microtubule-stabilizing conditions (a–k), coverslips were dipped in PBS and then PEEM (0.1 M PIPES, pH 6.9, 1 mM MgSO₄, 0.1 mM EDTA and 2 mM EGTA) and then placed in dishes containing PEEM plus 0.5% Triton X-100 (Pierce), 1 mM dithiothreitol and a mixture of protease inhibitors described previously, all at 37°C. After 30 s, coverslips were blotted dry and plunged immediately into −80°C methanol and 1% paraformaldehyde and placed into a freezer at −20°C for 30 min. For microtubule-destabilizing conditions (l–n), cells were extracted at 4°C for 5 min in a buffer containing 0.5% Triton X-100 and 80 μM CaCl₂, as described previously, and fixed as described above. Coverslips were rehydrated in TTBS (0.3% NaCl, 0.02% Triton (pH 7.3), 0.01% NaN₃, and 0.05% Tween-20). Coverslips were rinsed again in TTBS and blocked overnight in 10% normal goat serum in TTBS. Blocked coverslips were rinsed in PBS and incubated for 1 h at 37°C with a 50:50 mixture of 440.1 and 440.4 cell supernatants, rinsed in TTBS, 10 min each rinse, incubated for 1 h at 37°C with a 1.5 μl dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Boehringer) in 10% normal goat serum in PBS, rinsed as before, incubated for 1 h at 37°C with a 1.5 μl dilution of a rat anti-tubulin monoclonal YOLO3/34 (ref. 19) (Accurate Chemicals) in PBS, rinsed as before, incubated for 1 h at 37°C with a 1.5 μl dilution of rhodamine-labelled goat anti-rat IgG (Cappel) in 10% normal goat serum in PBS and rinsed as before. The first of the last three TTBS rinses contained 0.05% Tween-20. After this, 1.5 μl of a 1:200 dilution of a rat anti-tubulin monoclonal YOLO3/34 (ref. 19) (Accurate Chemicals) in PBS, added to a 10 μl dilution and the same solution plus 1% N-propyl-galactarate and observed under a Zeiss Axioscop microscope equipped with a Plan Neofluar 100×/NA 1.3 objective lens. Micrographs were taken with a TMAX 400 ASA black and white film (Kodak).

METHODS. CHO chromosomal isolates were isolated from vinblastine-arrested mitotic cells as described previously, except that the lysate buffer was slightly modified to 10 mM Pipes, 5 mM MgCl₂, 1 mM EGTA, pH 6.9 (PME) plus 0.1% digitonin. These chromosomal isolates had little or no endogenous tubulin associated with the kinetochore. Chromosomes prepared in this way from CHO cells that were incubated 16 h with 0.1 μg/ml 1 μc ml⁻¹ colcemid (Sigma) instead of vinblastine, have kinetochore-associated tubulin staining, indicating that isolated CHO chromosomes were prepared using a procedure slightly modified from that described above. Mitotic CHO cells were collected by mitotic shake-off from unchronized log-phase primary CEF culture provided by L. Tietz (University of California, San Francisco). The fibroblasts were gently lysed with one pass through a 18-gauge needle and spun through a 30% glycerol cushion in PME. The gentler technique was necessary to preserve the dynein staining of these chromosomes. Isolated chromosomes were fixed in 0.5% paraformaldehyde (Ted Pella, Inc.) for 10 min at 20°C, pelleted onto coverslips and post-fixed in −20°C methanol. Coverslips were blocked for 5 min in 1% BSA (20°C) and then incubated for 30 min at room temperature with 440.4 (full strength) or 70.1 (diluted 1:5 in 1% BSA) rinsed in TTBS, and double labelled with either rat anti-tubulin monoclonal antibody YOLO3/34 (ref. 19) or CREST anti-kinetochore sera (provided by F. McKeon, Harvard Medical School) rinsed as before and mounted in 0.02% p-diamino-benzene (Sigma) in glycerol–Tris pH 8.2. Coverslips were observed with an Olympus SPlan Apo 60× objective lens mounted on a Zeiss Photomicroscope III. Micrographs were taken with hyperred Technical Pan film (Kodak) and developed in HC110, dilution D.
cytoplasmic dynein antibodies (Fig. 3). This result strongly suggests that dynein is localized on kinetochore microtubules during metaphase and anaphase, but does not exclude the possibility that it is also localized on polar microtubules.

To demonstrate that our results were not limited to CEF cells, we used several of our antibodies to stain PtK1 cells, a marsupial (rat kangaroo) kidney cell line. The monoclonal antibody against the 70K polypeptide (70.1) and the goat polyclonal antiseraum cross-react with mammalian cytoplasmic dynein (data not shown). The similarity of the staining pattern to the fibroblasts and the PtK1 cells (data not shown) suggests that cytoplasmic dynein localization to the spindle is not unique to chick fibroblasts.

Because cytoplasmic dynein may be involved in chromosome movement, we stained isolated chromosomes from CEF cells with monoclonals 440.1, 440.4, 150.1 and 70.1, finding that the staining co-localizes with kinetochore staining, using CREST serum

Because cytoplasmic dynein is at the kinetochore of chromosomes in dividing cells. Although antibodies to axonemal dynein from sea urchin bind to the spindle in sea urchin eggs, localization of cytoplasmic dynein to the spindle has been reported previously. Immunolocalization of cytoplasmic dynein to the mitotic spindle in both vertebrate cells (see also Pfarr et al., this issue) and the nematode Caenorhabditis elegans (R. J. Lye et al., personal communication), raises the possibility that the enzyme plays a part in mitosis. These light-microscope studies do not identify the site of force generation, but indicate that in mitotic cells, cytoplasmic dynein is bound to kinetochore microtubules and to the kinetochore itself. Although cytoplasmic dynein is not localized exclusively on the kinetochore and kinetochore microtubules, it might be involved in the minus-end-directed movement of chromosomes during anaphase A and other stages of mitosis

It is apparent that there is a redistribution of cytoplasmic dynein at the onset of mitosis. The differences in the staining intensity between extracted interphase and mitotic cells supports the notion that dynein associates with a specific set of microtubules during mitosis, particularly those in the central portion of the spindle. One explanation for the regulation of this interaction could be a cell-cycle-dependent post-translational modification of cytoplasmic dynein or an activation of a dynein-binding protein that alters the affinity of dynein for spindle microtubules.

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The relationship of green algae to land plants has greatly interested botanists for more than a century. In recent years, several characters, particularly ultrastructural ones, have been used to define a green algal group (Charophyceae) from which land plants are thought to have arisen (refs 1–3, but see ref. 4). Here we provide the first molecular genetic evidence in support of the charophyan origin of land plants. Group II introns have previously been found in both the RNAa and RNAe genes of all land plant chloroplast DNAs examined, whereas all algae and bacteria examined have uninterrupted genes

The tRNAa and tRNAe genes are typically located in the transcribed spacer between the 16S and 23S rRNA genes in chloroplasts and bacteria

FIG. 1. Comparison of RNA spacer regions of Coleochaete orbicularis and Marchantia polymorpha chloroplast DNAs. Nucleotide sequence of 2,305 bp of a 3.2 kb HindII fragment from Coleochaete orbicularis chloroplast DNA cloned into plasmid (pBS, Stratagene) was determined using a combination of subclones and synthetic primers. Sequencing reactions with both dGTP and dITP (Sequenase) produced a series of overlapping sequences using double-stranded DNA. The entire sequence has been deposited with GenBank and is also available from the authors.