A cytoplasmic dynein required for mitotic aster formation in vivo

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Accepted 2 July; published on WWW 13 August 1998

SUMMARY

An astral pulling force helps to elongate the mitotic spindle in the filamentous ascomycete, Nectria haematococca. Evidence is mounting that dynein is required for the formation of mitotic spindles and asters. Obviously, this would be an important mitotic function of dynein, since it would be a prerequisite for astral force to be applied to a spindle pole. Missing from the evidence for such a role of dynein in aster formation, however, has been a dynein mutant lacking mitotic asters. To determine whether or not cytoplasmic dynein is involved in mitotic aster formation in N. haematococca, a dynein-deficient mutant was made. Immunocytochemistry visualized few or no mitotic astral microtubules in the mutant cells, and studies of living cells confirmed the veracity of this result by revealing the absence of mitotic aster functions in vivo: intra-astral motility of membranous organelles was not apparent; the rate and extent of spindle elongation during anaphase B were reduced; and spindle pole body separation almost stopped when the anaphase B spindle in the mutant was cut by a laser microbeam, demonstrating unequivocally that no astral pulling force was present. These unique results not only provide a demonstration that cytoplasmic dynein is required for the formation of mitotic asters in N. haematococca; they also represent the first report of mitotic phenotypes in a dynein mutant of any filamentous fungus and the first cytoplasmic dynein mutant of any organism whose mitotic phenotypes demonstrate the requirement of cytoplasmic dynein for aster formation in vivo.

Key words: Anaphase, Aster, Dynein, Microtubule, Mitosis, Spindle, Spindle pole body

INTRODUCTION

Cytoplasmic dynein is a microtubule (MT)-associated motor protein involved in mitotic movements (Barton and Goldstien, 1996; Pfarr et al., 1990; Schröer, 1994; Steuer et al., 1990), nuclear migration in the filamentous fungi Aspergillus nidulans (Xiang et al., 1994, 1995) and Neurospora crassa (Bruno et al., 1996; Plamann et al., 1994), and mitotic spindle orientation and astral MT dynamics in the yeast Saccharomyces cerevisiae (Carminati and Stearns, 1997; Ehshel et al., 1993; Li et al., 1993; Shaw et al., 1997). However, the various roles of cytoplasmic dynein in mitosis have not yet been fully elucidated and/or demonstrated (Holzbaur and Vale, 1994).

The spindle pole body (SPB) is a nucleus-associated microtubule-organizing center involved in mitosis and nuclear motility in fungi (Aist, 1995; Hagan and Yanagida, 1997). Mitotic anaphase in the filamentous ascomycete, Nectria haematococca MP VI, occurs as two sequential stages, anaphase A and anaphase B (Aist and Bayles, 1988), as defined by Inoué and Ritter (1975). Laser microbeam experiments and treatment with MT-depolymerizing drugs showed that astral MTs of N. haematococca are involved in a pulling force that is transmitted to the SPB during anaphase B and helps to elongate the mitotic spindle (Aist and Berns, 1981; Aist et al., 1991). Recent experimental research with animal cell systems (Compton, 1998; Echeverri et al., 1996; Gaglio et al., 1996, 1997; Heald et al., 1996; Merdes and Cleveland, 1997; Merdes et al., 1996; Rodionov and Borisy, 1997; Vaisberg et al., 1993; Verde et al., 1991) has indicated that dynein is required for the formation and maintenance of mitotic spindle poles and asters, a prerequisite for astral force to be applied to a spindle pole. However, one key element of in vivo evidence for such a role of dynein in aster formation that has, until now, been missing (cf. Compton, 1998; Merdes and Cleveland, 1997), is a dynein-deficient mutant that fails to form mitotic asters.

To determine if cytoplasmic dynein heavy chain (CDHC) is required for the formation of mitotic asters in N. haematococca, we created a CDHC-deficient mutant, Cu1, from wild-type strain T213 by inserting a selectable marker into the central motor domain of the CDHC gene, DHC1 (Inoue et al., 1998). In this paper we report the first mitotic phenotypes of a dynein mutant of any filamentous fungus and the first cytoplasmic dynein mutant of any organism whose mitotic phenotypes demonstrate the requirement of cytoplasmic dynein for aster formation in vivo. A brief summary of these results has been published (Inoue et al., 1997).
MATERIALS AND METHODS

Molecular manipulations
Detailed methods and additional molecular results have been published (Inoue et al., 1998). DNA gel blot analysis indicated that there is only one CDHC gene in N. haematococca. To create the CDHC-deficient mutant (Cu1), the CDHC gene, \textit{DHC1}, in wild-type isolate T213 was disrupted by transformation with a vector carrying a portion of \textit{DHC1} interrupted by a gene, \textit{hygB}, for resistance to hygromycin B. The control transformant, S01-2, was created by integration of the same transformation vector at an ectopic site, leaving \textit{DHC1} intact.

Microscopy and microscopic analyses
Procedures for microscopic analyses were similar to those previously described (Aist and Bayles, 1988; Aist et al., 1991; Wu et al., 1998). Briefly, cultures were grown on glass microscope slides coated with yeast extract-glucose (YE)-Gelrite medium and videotaped at ×10,000 magnification using phase-contrast optics and real-time image processing. Videotaped images were captured and processed with the computer program Image-Pro Plus (Media Cybernetics, Silver Spring, MD) and printed with a Codonics NP-1600 Photographic Network Printer (Codonics, Middleburg Heights, OH). A customized software program designed by W. Schubarg (Empire Imaging Systems, Cicero, NY) was used to acquire mitotic data from videotapes. From the mitotic data spreadsheets, plots of spindle elongation and movements of the SPBs and mitotic apparatus of each analyzed mitosis were created by Sigma Plot for Windows\textsuperscript{TM} (SPSS Inc., Chicago, IL).

Laser microsurgery
Cultures were grown for 5-6 days on glass microscope slides coated with YE-Gelrite medium. We conducted the laser microsurgery experiments in the Biological Microscopy and Image Reconstruction Resource at the Wadsworth Center in Albany, NY. Development of the differential interference contrast-based light microscopic system we used for laser microsurgery was described elsewhere (Cole et al., 1995). A Q-switched, pulsed (10 Hz), neodymium-yttrium-aluminum-garnet laser was used for laser microsurgery. After preliminary experiments, we decided to use a laser power between 4.5 and 6.0 mJ, which consistently broke the early anaphase B spindle without apparent, short-term, deleterious effects on the cells. One to three pulse trains of 0.5-1.0 seconds each were required to cut the spindles, and comparable irradiations were given to the nucleoplasm (beside the spindle) in control cells. Each experiment was videotaped at ×12,000 magnification with a Nikon ×100 PlanApo objective lens and real-time image processing using the computer program Image 1 (Universal Imaging Corp., West Chester, PA). Images were captured from videotapes, and selected linear measurements were calculated as described above under Microscopy and microscopic analyses.

MT immunocytochemistry
Tubulin immunocytochemistry has been shown to be an effective approach to visualization of MTs of the mitotic apparatus (asters and spindle) in yeast when compared to either electron microscopy or GFP-tubulin fluorescence (Carminati and Stearns, 1997). Moreover, when applied to the mitotic apparatus of \textit{N. haematococca} (Aist et al., 1991), immunocytochemistry gave results corresponding closely to those obtained using freeze-substitution fixation followed by electron microscopy of serial thin-sections and computerized three-dimensional reconstruction methodologies (Jensen et al., 1991). In addition to astral MTs, non-astral cytoplasmic MTs in \textit{N. haematococca} are readily visualized by this procedure (Inoue et al., 1998; Wu et al., 1998).

In the present study, two day-old germlings on YEG-Gelrite-coated glass slides were used. Immunofluorescent MT staining was performed following the protocol of Aist et al. (1991). The optics were a Zeiss ×63 Neofluar objective (NA 1.25) and a ×16 projection lens.

RESULTS

Immunocytochemistry of MTs (Fig. 1) revealed prominent spindles and extensive asters of all mitotic nuclei in the controls, wild-type T213 and isolate S01-2 (Fig. 1C,E,G). Although clear staining of spindles (Fig. 1I,K,M,O) was often of non-astral cytoplasmic MTs in the same mitotic cells (not illustrated), was seen in the mutant, no prominent asters were observed in the 31 anaphase B nuclei of mutant cells we videotaped (Fig. 1M and O). In a small minority (7 of 31) of these mitoses in the mutant, one or two thin, 2-6 µm long MTs or MT bundles were observed projecting from one of the two SPBs at anaphase B. From these unique results we inferred that little or no mitotic aster was formed in the CDHC-deficient mutant and that CDHC has as essential role in the formation of asters in \textit{N. haematococca}.

This lack of prominent asters in fixed and stained cells of the mutant was confirmed by several unique observations and analyses of aster function performed with living cells. First, rapid migrations of small organelles toward the SPB and back again within the astral regions (intra-astral motility; Aist and Bayles, 1991a) were numerous and easily observed during anaphase B in control cells, but none was observed in the mutant, despite extensive efforts to detect them by carefully reviewing videotaped sequences of 26 mitoses. Instead, small vesicles and lipid bodies moved rapidly and freely, by Brownian motion, in the presumed astral regions of the mutant, apparently due to the absence of astral MTs. Second, the rate and extent of spindle elongation during anaphase B of the mutant were reduced, with the elongation rate being only about half that of the controls (Table 1). This slower rate of spindle elongation in the mutant is consistent with previous observations (Aist and Bayles, 1991b) in which a similarly slow spindle elongation rate was inferred to be caused by a momentary absence of the astral pulling force during spindle bending episodes in wild type. Third, the rate of migration (Table 1) and the frequency of oscillations (not illustrated) of the mitotic apparatus during anaphase B in the mutant were reduced, reflecting absence of the astral pulling force (Aist and Bayles, 1988). And fourth, we performed laser microsurgery on the anaphase B spindle of the mutant, using the ectopic transformant (S01-2) as a control. When the spindle of the control was cut by laser irradiation, the SPBs began to move.

Table 1. In vivo characteristics of anaphase B spindles of wild-type (T213), ectopic transformant-control (S01-2) and dynein mutant (Cu1) strains of \textit{N. haematococca}*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Final spindle length (µm)</th>
<th>Spindle elongation rate (µm/minute)</th>
<th>Rate of forward migration (µm/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T213</td>
<td>(&gt;20.00)§</td>
<td>7.05 (±1.26) a</td>
<td>4.00 (±2.11) a</td>
</tr>
<tr>
<td>Cu1</td>
<td>15.00 (±2.42)</td>
<td>3.58 (±0.85) b</td>
<td>0.80 (±0.58) b</td>
</tr>
<tr>
<td>S01-2</td>
<td>(&gt;20.00)§</td>
<td>7.83 (±1.16) a</td>
<td>2.65 (±1.33) a</td>
</tr>
</tbody>
</table>

*Data are means (±1 s.d.). Values in each column not followed by the same letter were significantly different (P<0.01) by the two-sample t-test.

‡The mitotic apparatus in hyphal tip cells usually migrates toward the tip. Since the final spindle length of T213 and S01-2 was usually more than 20 µm, a complete data set could not be collected because most SPBs were off the video screen at the end of anaphase B.
apart immediately after irradiation, separating three times as rapidly as those of the nucleoplasm-irradiated controls (Figs 2 and 3). Presumably, this was the rate of movement that the asters generated on their own, without limitation by the spindle. This result confirmed that a strong astral pulling force exists in control cells, counterbalanced by the intact spindle which limits the rate of SPB separation, as first reported by Aist and Berns (1981). In contrast, SPB separation almost stopped in the CDHC-deficient mutant when the spindle was cut with the laser microbeam (Figs 2 and 3), showing conclusively that without cytoplasmic dynein there was no astral pulling force; because the asters were absent, only the spindle pushing force was available to separate the SPBs when the spindle was left intact. Thus, cytoplasmic dynein is required for the astral pulling force to be manifested during anaphase B in vivo, a result that is consistent with the absence of asters in the mutant. The rate of SPB separation (a measure of the spindle elongation rate) in the Cu1 nucleoplasm-irradiated controls (Table 2) was almost the same as the spindle elongation rate we obtained in unirradiated cells (Table 1), showing that the laser irradiation itself did not have a significant affect on the spindle elongation/SPB separation rate.

Laser microsurgery on the anaphase B spindle of the CDHC-deficient mutant made it possible to observe a unique behavior of the severed spindle segments, because, in contrast to wild type, the SPBs in the mutant became almost stationary after the spindle was cut, rather than migrating rapidly apart and out of view (Fig. 2). The cut spindle segments first rotated slightly in opposite directions (Fig. 2C), and then each elongated toward the opposite SPB until their free ends had passed each other (Fig. 2D). The average elongation rate of the cut spindle

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**Fig. 1.** Mitotic nuclei of the ectopic transformant control (A,B,E-H), the wild-type control (C,D) and the dynein mutant (I-P) stained with FITC-labeled antibody (for MTs) (A,C,E,G,I,K,M,O) and with 4′,6-diamidino-2-phenylindole (for chromatin) (B,D,F,H,J,L,N,P). As, aster; IP, interphase nucleus. Triangles indicate the approximate positions of SPBs. Note that no aster is visualized in these typical anaphase A (K) and anaphase B (M and O) nuclei in the mutant, whereas developing asters were often visualized at anaphase A (C) and prominent asters were consistently visualized at anaphase B (E and G) in the controls. The left end of the spindle in M is slightly out of the plane of focus, accounting for the diminishing fluorescence signal from the spindle near the left SPB. Bar, 5 μm.
segments was 4.32 (± 2.04 s.d.) μm/minute (n=6). Eventually, the cut spindle segments rotated back until they were parallel to each other, and then they appeared to re-join, forming what we are calling apparently intact spindles (Fig. 2E). As revealed by careful still-frame reviews of the videotapes, these apparently intact spindles were formed when the overlapping, free ends of the elongated, parallel cut spindle segments became associated laterally, giving the initial impression of a regenerated spindle. Apparently intact spindles are nonfunctional, as they do not achieve further SPB separation.

Mitosis in the wild-type (T213) and ectopic transformant-control (SO1-2) strains was similar, indicating that transformation itself had no appreciable affect on mitosis. Moreover, the mitotic phenotypes caused by CDHC deficiency in Cu1 were restricted to anaphase B: mitosis occurred normally from prophase to anaphase A (Fig. 4), except for a marked diminution of oscillatory movements and rotations of the mitotic apparatus which was obvious during review of the video tapes. The duration (23 to 29 seconds), net spindle elongation (approx. 2.0 μm) and rate of spindle elongation (3.3 to 4.2 μm/minute) during anaphase A in the mutant were similar to those in the controls. The separation distance of incipient daughter nuclei during anaphase B was diminished in the mutant (Fig. 4C and F; Table 1).

DISCUSSION
Role of dynein in aster formation
We have not ruled out the possibility that in the absence of functional cytoplasmic dynein, the astral MTs in our dynein mutant are preferentially unstable and therefore selectively sensitive to depolymerization during fixation. Nevertheless, our tubulin immunocytochemistry protocol has been shown to be reliable, our MT staining results were clear and consistent, and the pattern of organelle movements in all astral regions of the mutant cells was indicative of the absence of astral MTs.
Therefore, the best interpretation of our results is that mitotic cells of our dynein mutant are unable to form mitotic asters, which points to a role for dynein in aster formation.

Our previous studies and those of others provide a context in which possible mechanisms of this dynein function in *N. haematococca* may be envisaged. Astral MTs polymerize from the SPBs during anaphase A and B and elongate into the cytoplasm to form roughly cone-shaped arrays (Aist and Bayles, 1991c; Aist et al., 1991). These asters are composed of numerous MTs of variable length, some of which apparently are attached to the SPBs (polar MTs), but most of which are not attached directly to the SPBs (free MTs). Thus, we inferred that the astral MTs have a rapid turn over rate and exhibit dynamic instability (Aist and Bayles, 1991c). Many of the astral MTs are bundled, being cross-linked by numerous bridges (Jensen et al., 1991).

A few of the astral MTs terminate in the cell cortex near the plasma membrane in a tuft of fine, fibrillo-granular material, suggestive of a functional interaction of the astral MT plus ends with some as yet unidentified component of the cortex (Aist and Bayles, 1991c; Aist and Berns, 1981). Dynactin has been found not only in animal cells, but also in the filamentous ascomycete *N. crassa* (Plamann et al., 1994; Tinsley et al., 1996). It can bind to both dynein and MTs and apparently functions to anchor dynein to subcellular binding sites on various organelles (Vallee and Sheetz, 1996).

Based on the preceding information, we suggest four possible mechanisms for the participation of a cytoplasmic dynein-dynactin complex in aster formation in wild-type *N. haematococca* (Fig. 5). Such a complex may bridge polar and free MTs to each other, thus holding the free MTs in the asters (Fig. 5A). Cross bridging may involve additional free MTs as well, to form small MT bundles that are held similarly in the asters (Fig. 5B). As a minus end-directed motor (Vallee, 1993),

![Fig. 3. Plots of SPB separation of the dynein mutant, Cu1, and the ectopic transformant-control, S01-2, following laser microbeam cutting of the spindle (CUT) or comparable control irradiation of the nucleoplasm (Control) at early anaphase B in *N. haematococca*. Note that SPB separation was accelerated two- to threefold in S01-2 when the spindle was cut. In contrast, SPB separation almost stopped when the spindle was cut in Cu1, demonstrating the absence of the astral pulling force in the dynein mutant.](image3)

![Fig. 4. Phase-contrast, time-lapse videomicrographs of representative mitoses in living cells of wild-type T213 (A,B,C) and the CDHC-deficient mutant Cu1 (D,E,F). Elapsed time (minutes and seconds) is shown in the upper right corner of each panel. Ch, chromosomes; NE, nuclear envelope; Sp, spindle; V, vacuole. Triangles indicate the positions of SPBs. Note that chromosomes were clustered on the spindle during metaphase in both isolates (A and D). The right end of the spindle in D was momentarily out of the plane of focus. Chromatids were then segregated to the poles during anaphase A in both isolates, where they formed dark clusters near the SPBs at the beginning of anaphase B (B and E). These chromatid clusters momentarily obscured the SPBs from view. There was no discernible difference between wild type and mutant up to the beginning of anaphase B, except for a marked diminution of oscillatory and rotatory movements of the mitotic apparatus in the mutant. During anaphase B (Aist and Bayles, 1988) in both isolates, the SPBs were further separated from each other as the spindle elongated, and the nuclear envelope constricted behind each incipient daughter nucleus, which appeared as small (2-3 µm wide), pear-shaped organelles, each attached to its SPB (triangles, C and E). The spindle eventually disappeared, at the end of anaphase B (C and F). Compared to wild type (C), separation of the SPBs during anaphase B was less extensive in the mutant (F). Bar, 5 µm.](image4)
Counterparts of the aster-less mitotic apparatus

This is the first report of any CDHC-deficient transformant that lacks mitotic asters. By contrast, CDHC-deficient mutants of yeast retain the ability to form mitotic asters (Eshel et al., 1993; Li et al., 1993; Yeh et al., 1995) and the astral MTs are longer than in wild type (Carminati and Stearns, 1997; Cottingham and Hoyt, 1997). Aster-less mitotic apparatuses were found also in S. cerevisiae kip2Δ, a kinesin-related protein mutant (Cottingham and Hoyt, 1997) and tub2-401, a β-tubulin mutant (Sullivan and Huffaker, 1992). It is now clear that several different motor proteins are involved in aster formation and the dynamics of astral MTs (Compton, 1998).

The finding of aster-less mitotic apparatuses in our dynein mutant is consistent with results obtained by others using Xenopus and mammalian cells and cell-free preparations (Gagliò et al., 1996; Heald et al., 1996; Merdes et al., 1996; Vaisberg et al., 1993; Verde et al., 1991) in which astral and spindle MTs were not focused properly in the absence of functional CDHC, as well as with those from animal cells overexpressing a subunit of dynactin, in which ‘…few or no astral microtubules were visible’ (Echeverri et al., 1996). A dynein-like motor was shown to be necessary also for the formation of radial arrays of cytoplasmic MTs in fragments of fish melanophore cells (Rodionov and Borisy, 1997). As the first reported case of a CDHC-deficient mutant, in any organism, that is unable to form mitotic asters, our work supplies a key element that has been missing from the mounting evidence that cytoplasmic dynein has an essential role in aster formation (cf. Compton, 1998; Merdes and Cleveland, 1997). However, the spindle poles in our dynein-deficient mutant appeared to be normal, in contrast to the experimental results obtained with animal cells, but consistent with the results obtained with dynein mutants of the yeast, S. cerevisiae (Eshel et al., 1993; Li et al., 1993). In view of the disparate results mentioned above, we infer that cytoplasmic dynein may be more important in the formation of mitotic asters in the cells of filamentous fungi and higher eukaryotes (e.g. Nectria, Xenopus and mammalian cells) than in the cells of lower eukaryotes, such as Dictyostelium and Saccharomyces, in which its function may be limited more specifically to anchoring the plus ends of astral MTs.

cytoplasmic dynein associated with polar MTs would be expected to travel to the SPB. After arriving at the SPB, the dynein-dynactin complex could then tether polar MTs to the SPB by binding to the polar MTs via dynein and to a hypothetical tethering component of the SPB via dynactin (Fig. 5C). In yeast, cytoplasmic dynein is localized at the cell cortex (Yeh et al., 1995), and disruption of the dynein gene altered the dynamics of astral MTs (Carminati and Stearns, 1997). Furthermore, overexpression of a dynein-green fluorescent protein fusion stabilized astral MTs, producing longer MTs and making the asters persist longer (Shaw et al., 1997). Thus, it seems reasonable to speculate that a cytoplasmic dynein-dynactin complex could stabilize astral MTs in N. haematococca via interactions with their plus ends (Fig. 5D), thereby contributing to aster formation. Of course, further research will be required to determine if dynein is located in the asters, at the SPBs and/or in the cell cortex and to then distinguish among the four possible mechanisms depicted in this preliminary, hypothetical, working model.

Mechanics of anaphase B

Several important conclusions can now be drawn about the mechanics of anaphase B in N. haematococca, because of our present in vivo observations and experiments on mitosis in dynein mutant cells that are unable to form asters. First, the fact that the spindle elongates without the aid of the astral pulling force demonstrates, unequivocally, the presence of the spindle pushing force, which was only inferred previously from the dynamics of spindle bending episodes in wild-type cells (Aist and Bayles, 1991b). Second, our present laser microbeam results confirmed the absence of the astral pulling force and the presence of the spindle pushing force in the dynein mutant. Thus, these two forces are now firmly established as redundant mechanisms for separating the mitotic SPBs in wild-type N. haematococca. Third, our results are consistent with those obtained with other fungi.
in aster formation and function. Different mechanisms; conserved components.

REFERENCES


