Three-dimensional analysis of mitosis and cytokinesis in the binucleate parasite *Giardia intestinalis*

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Summary

In the binucleate parasite *Giardia intestinalis*, two diploid nuclei and essential cytoskeletal structures including eight flagella are duplicated and partitioned into two daughter cells during cell division. The mechanisms of mitosis and cytokinesis in the binucleate parasite *Giardia* are poorly resolved, yet have important implications for the maintenance of genetic heterozygosity. To articulate the mechanism of mitosis and the plane of cell division, we used three-dimensional deconvolution microscopy of each stage of mitosis to monitor the spatial relationships of conserved cytological markers to the mitotic spindles, the centromeres and the spindle poles. Using both light- and transmission electron microscopy, we determined that *Giardia* has a semi-open mitosis with two extranuclear spindles that access chromatin through polar openings in the nuclear membranes. In prophase, the nuclei migrate to the cell midline, followed by lateral chromosome segregation in anaphase. Taxol treatment results in lagging chromosomes and half-spindles. Our analysis supports a nuclear migration model of mitosis with lateral chromosome segregation in the left-right axis and cytokinesis along the longitudinal plane (perpendicular to the spindles), ensuring that each daughter inherits one copy of each parental nucleus with mirror image symmetry. Fluorescence in situ hybridization (FISH) to an episomal plasmid confirms that the nuclei remain separate and are inherited with mirror image symmetry.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/23/4889/DC1

Key words: *Giardia intestinalis*, Mitosis, Cytokinesis, Spindle, Basal body, Centromere

Introduction

*Giardia intestinalis*, a widespread zoonotic intestinal parasite, has two life-cycle stages: a binucleate, double diploid flagellate or trophozoite form that attaches to the intestinal microvilli, and an infectious cyst form that persists in the environment (Adam, 2001; Gillin et al., 1996). Over several million cases of malabsorptive diarrhea by giardiasis are estimated to occur annually worldwide, and waterborne outbreaks of giardiasis are frequent in areas where unfiltered waters are routinely contaminated. (Savioli et al., 2006). Although *Giardia* is well known in terms of disease (Adam, 2001; Savioli et al., 2006) and has received much recent attention due to evolutionary controversies (Baldauf, 2003; Baldauf et al., 2000; Best et al., 2004; Ciccarelli et al., 2006; Dacks et al., 2002; Graczyk, 2005; Knight, 2004; Sogin et al., 1989), there remains little knowledge of the assembly and division of the complex giardial cytoskeletal systems required for the life cycle of the parasite, including the mechanism of mitosis.

Like all diplomonads, *Giardia* has two diploid nuclei (2n=10), which are both transcriptionally active and identical in DNA content (Adam et al., 1988; Kabnick and Peattie, 1990; Wiesehahn et al., 1984; Yu et al., 2002). In addition, *Giardia* is bilaterally symmetrical and possesses a complex and distinctive microtubule cytoskeleton that establishes anterior-posterior, left-right and dorsal-ventral polarity and, importantly, plays a major role in its virulence (Elmendorf et al., 2003). The microtubule cytoskeleton of the trophozoite, or intestinal form, is characterized by four main elements: eight flagellar axonemes and basal bodies, the ventral disc, the funis and the median body (Fig. 1A,B). *Giardia* is unique among diplomonads in that it possesses the ventral disc, a novel organelle composed of an overlapping spiral lamella of microtubules that mediates attachment to the intestinal microvilli (or a laboratory substrate), most likely by a suction-based mechanism (Hansen et al., 2006).

To complete cell division, the complex giardial cell must duplicate and partition both diploid nuclei as well as the multiple cytoskeletal structures. Thus, a detailed analysis of mitosis in *Giardia* at both the ultrastructural and molecular level is needed to resolve two intriguing questions. First, how does *Giardia* coordinate the division of two equivalent nuclei and a complex microtubule cytoskeleton? Second, how conserved is the mechanism of mitosis in a highly divergent and putatively early branching eukaryote? Understanding chromosome segregation in *Giardia*, however, has been hampered principally by the lack of cytological descriptions of intermediate stages of mitosis, including the inability to identify the mitotic spindle (Solari et al., 2003). Several prior studies have sought to identify the stages of mitosis using primarily light microscopy and chromatin staining, yet have not described a mitotic spindle (Cerva and Nohynkova, 1992; Filice, 1952). Furthermore, recent debate concerning the mechanism of giardial cell division has lead to proposals of unconventional mechanisms of chromosome segregation,

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Fig. 1. *Giardia* has two equivalent nuclei and a complex microtubule cytoskeleton. (A) The microtubule cytoskeleton of the trophozoite is characterized by eight flagellar axonemes and basal bodies (afl, anterior; c, caudal; pfl, posterior; vfl, ventral), the ventral adhesive disc (vdd), the funis (fn) and the median body (mb) (diagramed in A). All basal bodies are between the nuclei. (B) Anti-tubulin labeling reveals the microtubule arrays, including the eight flagella, and median body. The ventral disk is more weakly stained than the other structures. Although images of *Giardia* are frequently presented as two-dimensional projections in dorsal perspective as in A, describing cell division in *Giardia* requires analysis of cell morphology in three dimensions. (C) The cell is shown in 3D from a side angle. The spatial positions of the four nuclei following nuclear division are shown arrayed along the left-right (L-R) and dorsal-ventral (D-V) axes. Three possible planes of cytokinesis are represented in C; longitudinal [anterior-posterior (A-P)], transverse (L-R) and frontal (D-V). The dorsal-posterior nuclei are marked with asterisks in C,D.

This study is the first to observe dual mitotic spindles, to confirm the role of centrin directly at the spindle poles and to infer spindle attachment to the chromosomes at the centromere (using the centromere histone variant cenH3). By our detailed analysis of mitotic stages using 3D deconvolution light microscopy and transmission electron microscopy (TEM), we find that mitosis is semi-open, with two extranuclear central spindles and microtubules that enter the nuclei through polar openings in the nuclear envelopes. Chromosomes are segregated along the left-right (L-R) axis, and cytokinesis occurs along the longitudinal axis – perpendicular to the spindle – suggesting that daughter cells inherit one copy of each parent nucleus.

By monitoring these cytological markers at all stages of mitosis and cytokinesis, we present a model of cell division in *Giardia* that: (1) establishes both the spatial orientation and the timeline of chromosome segregation (mitosis) and cytokinesis; (2) illustrates the timing of the duplication of cytoskeletal structures including the median body, the ventral disc and the eight flagella; and (3) accounts for the fate of each nucleus during cell division. This model of cell division can explain prior studies on nuclear partitioning during cytokinesis, albeit with an alternative mode of cytokinesis than that originally proposed (Ghosh et al., 2001).

**Results**

Traditionally, identifying individual mitotic stages in *Giardia* has been difficult due to the inability to generate synchronized cultures. Therefore, we first developed a method for enriching cultures of *Giardia* for mitotic cells (supplementary material Table 1) followed by indirect immunofluorescence of key cytological markers to visualize changes in the microtubule cytoskeleton throughout mitosis. To describe mitotic cells we immunostained mitotic spindles (anti-tubulin) and the spindle poles (anti-centrin), visualized green fluorescent protein (GFP)-tagged centromeres (cenH3::GFP) and tracked hallmark features of the spindle during mitosis using 3D deconvolution microscopy (Materials and Methods). Between 50-100 cells were analyzed for each mitotic stage; representative images are presented in Fig. 2.

These analyses allow the establishment of a timeline of the crucial events of mitosis and the determination of the plane of nuclear division and cytokinesis. Although we emphasize the dynamics of chromosome segregation during mitosis at high levels of resolution, we also present a timeline of the duplication and partitioning of major cytoskeletal elements including the eight flagella, the median body and the ventral disc, albeit in less detail.

**Interphase**

Microtubule cytoskeleton: *Giardia* trophozoites have an elaborate microtubule cytoskeleton, consisting of four major structural microtubule arrays: a ventral disc, flagellar axonemes with basal bodies, the funis and the median body (an enigmatic microtubular array of unknown function) (see Fig. 1A,B) (see also Elmendorf et al., 2003). In the images presented here, the ventral disc is weakly stained relative to the other microtubule organelles.

Chromosomes: in interphase, chromatin is decondensed, as
Mitosis in *Giardia* indicated by the uniform fluorescence of 4,6-diamino-2-phenylindole (DAPI)-stained nuclei (Fig. 2A,D). Although eukaryotic centromeric sequences vary greatly at the nucleotide level, centromeric nucleosomes contain the specialized histone H3 variant cenH3 (Malik and Henikoff, 2003). To visualize the centromeres of *Giardia* chromosomes, we identified the cenH3 homolog in *Giardia* and constructed a GFP fusion protein (S.C.D. and W.Z.C., unpublished data). In interphase, cenH3::GFP foci were seen throughout the DAPI-stained nuclei, indicating that the cenH3::GFP marks discrete centromeric loci on the chromosomes (Fig. 2A).

Basal bodies: to determine the role of basal bodies in organizing the giardial mitotic spindles, we monitored the movement of flagellar basal bodies during interphase and mitosis using anti-centrin immunostaining. The basal bodies of all eight flagella are located between the two adjacent nuclei in the anterior region of the cell. In interphase trophozoites, we observed that centrin localized to two clusters between the two nuclei, colocalizing with the flagellar basal bodies (Fig. 2D) as has been shown previously (Belhadri, 1995; Correa et al., 2004; Meng et al., 1996).

**Prophase**
Mitotic prophase is characterized by extensive chromatin condensation and the nucleation of spindle microtubules. As in most eukaryotes, we found that individual giardial chromosomes could be resolved following chromatin condensation in mitotic prophase (Fig. 2B). In prophase, each chromosome contained a single cenH3::GFP focus, indicating that each of the ten chromosomes in each nucleus has a single centromeric locus. Spindle microtubule nucleation was also initiated during prophase: microtubules first appeared between the two nuclei near the flagellar basal bodies and extended around each nucleus individually (Fig. 2B).

An unanticipated repositioning of the nuclei occurred throughout prophase. The two nuclei migrated to the cell midline one nucleus on top of the other along the dorsal-ventral axis, with the ventral nucleus slightly anterior to the dorsal nucleus (supplementary material Movie 1). Three-dimensional reconstructions of these cells showed the two nuclei remained separate from one another (supplementary material Movie 1). At the completion of nuclear migration individual chromosomes could be distinguished. During nuclear migration the spindle...
microtubules continued to elongate, encompassing each nucleus by the end of prophase.

Duplication of the centrin foci also occurred during prophase. At the onset of prophase and spindle assembly the number of centrin foci increased from two to four as the result of either duplication or separation of the basal bodies (Fig. 2E).

During nuclear migration the centrin foci were present at the sites of microtubule nucleation. These foci moved around the periphery of the nucleus as the spindle microtubules continued to elongate and the nuclei migrated to the center. When the two nuclei were stacked, two centrin foci, each with its own complement of microtubules, were positioned on opposite sides of each nucleus.

Metaphase

Upon completion of nuclear migration, the microtubules surrounding each nucleus formed two independent bipolar spindles, stacked in the dorsal-ventral plane (Fig. 2C,F). The opposing poles of each spindle were oriented along the left-right axis of the cell. The chromatin was clustered tightly in the center of each spindle axis so that individual chromosomes were not visible (Fig. 2C). We were unable to observe a canonical metaphase alignment of centromeres along a metaphase plate. At this time, centrin localized to each of the four spindle poles (Fig. 2F).

Anaphase A and B

Chromosome segregation in both nuclei occurred in two stages: chromatid segregation to the spindle poles in anaphase A, followed by spindle elongation along the left-right axis of the cell in anaphase B (Fig. 2G). In Giardia, anaphase A initiates first in one nucleus (data not shown), however anaphase B occurs simultaneously between the two nuclei. As a result of both nuclear migration and lateral chromosome segregation, the sister chromatids from each nucleus were segregated to opposite sides (L-R) of the cell. At the completion of anaphase, nuclei of different parental origin reside on opposite sides of the cell with the daughters of the dorsal nucleus remaining dorsal and slightly posterior with respect to the daughter nuclei of the ventral nucleus.

During anaphase A, the cenH3::GFP localized to the leading edge (near the spindle pole) of the segregating DNA, a behavior characteristic of kinetochore attachment to microtubules. The cenH3::GFP foci remained tightly clustered together at the spindle poles during anaphase B (Fig. 2G). This distribution pattern implicates centromeres as the site of microtubule attachment during chromosome segregation in Giardia as in other eukaryotes. Centrin foci remained at the spindle poles throughout anaphase A and anaphase B (Fig. 2J).

Telophase and cytokinesis

In telophase, a microtubule bundle with unfocused ends extended between the nuclei on each side of the cell replacing the bipolar spindle arrays (Fig. 2H). We interpret this structure as the remaining spindle microtubules following the loss of focused spindle poles. We are not able to rule out formation of this structure from de novo microtubule polymerization; however, a de novo origin is unlikely given that intermediate structures were not observed. The single cenH3::GFP foci observed in the daughter nuclei indicated that the centromeres remained clustered in telophase nuclei.

During telophase, the two centrin foci on each side of the nuclei moved from their anaphase position near the cell periphery to their position between each pair of nuclei as seen in interphase (Fig. 2K). By the onset of cytokinesis, the DNA was decondensed, cenH3::GFP foci were seen throughout the nucleus, the nuclei were adjacent to one another and all cytoskeletal structures were regenerated (described below).

To complete cytokinesis, a furrow formed at the anterior end of the cell creating a heart-shaped four nuclear stage cell (Fig. 2I,L). The furrow progressed from anterior to posterior, in the longitudinal plane, separating the left and right sides of the heart into the two daughter cells. As a result, each daughter cell inherits two nuclei each derived from one left and one right nucleus of the mother cell.

Patterns of nuclear inheritance using FISH of an episomal plasmid in mitotic nuclei

To determine the pattern of nuclear partitioning and inheritance, we used fluorescence in situ hybridization (FISH) to visualize an episomal plasmid in the cenH3::GFP strain, which is maintained by one nucleus in each Giardia trophozoite (Ghosh et al., 2001; Singer et al., 1998; Yu et al., 2002). In each cell, we observed the episomal plasmid FISH signal in only one nucleus (either the left or the right) as previously reported (Ghosh et al., 2001). Since a nucleus contains more than one episme, by using deconvolution microscopy we are able to detect multiple discrete FISH signals in the labeled nucleus. In prophase nuclei, during the late stages of nuclear migration, the FISH signal was observed only in one nucleus (see dorsal nucleus in Fig. 3B,B’). Given our previous observation that daughter nuclei from each

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**Fig. 3.** FISH to an episomal plasmid maintained in one nucleus shows nuclei are inherited with mirror image symmetry and that the nuclei never mix.

(A) Interphase; the FISH probe hybridizes to the episme contained in the right nucleus.

(B) In the metaphase nuclei, stacked on top of each other in the cell center, FISH signal is detected in the dorsal nucleus.

(B’’) Projection of the first eight z-sections, dorsal nucleus, of the cell seen in B contains the FISH signal. (B’’) Projection of the last eight z-sections, ventral nucleus, of the cell shown in B shows the FISH signal is excluded. (C) During cytokinesis, one nucleus in each daughter is labeled with mirror image symmetry between the two cells. Red, DAPI; green, FISH. Bars, 2 μm.
nucleus are segregated laterally, we would expect only one nucleus in each daughter cell to contain the episome. As predicted, in heart-shaped cells entering cytokinesis, only one nucleus in each future daughter cell showed the episomal plasmid FISH signal (Fig. 3C); the two cells showed mirror image symmetry of the labeled nuclei as has been previously reported (Ghosh et al., 2001).

Giardia has a semi-open mitosis

We used TEM to visualize spindle ultrastructure, to assess the integrity of the nuclear envelope during mitosis and to determine the identity of the spindle pole. TEM images of both early and late mitotic nuclei showed that the spindles are extranuclear and that the nuclear envelope remains intact throughout mitosis (Fig. 4A). Microtubules originating from each spindle pole form a sheath around the nucleus outside of the nuclear envelope. The nuclear envelope elongates with the spindle in anaphase B, extending between the spindle poles of fully elongated spindles. The persistence of the nuclear envelope throughout mitosis precludes mixing of the chromatin between nuclei. Furthermore, we did not observe fusion of the two nuclei at any mitotic stage.

Although the nuclear envelope is present throughout, this type of mitosis can be classified as semi-open. The right spindle pole (outlined by the box in Fig. 4A) is shown at greater magnification in Fig. 4B. Cytoplasmic microtubules from spindle poles passed through large openings in the nuclear membrane (Fig. 4B). In the late-stage (anaphase B) nuclei, the internal (presumably kinetochore) microtubules extended a few microns into the nucleus near the chromatin, seen as an electron-dense region near the pole (Fig. 4B).

We observed both basal bodies as well as centrin localization at each of the four spindle poles. Each spindle pole was associated with at least one axoneme (Fig. 4A,C). We imaged at least two basal bodies positioned at right angles to one another at each spindle pole: one basal body is in cross-section, whereas the second basal body with associated axoneme – at a more peripheral location relative to the spindle pole – is seen in longitudinal section (Fig. 4A,C). The basal body cross-section revealed a ring of doublet microtubules that lack a central microtubule pair (Fig. 4C), resembling the transition zone of a basal body as described in *Chlamydomonas* (O’Toole et al., 2003). Spindle microtubules radiated from one of the basal bodies (seen in cross-section) in proximity to spindle poles (Fig. 4C). In telophase the flagellar basal bodies were positioned between the two daughter nuclei on each side of the cell (Fig. 4D).

Microtubule stabilization disrupts mitotic spindles and causes chromosome missegregation

Prior studies have suggested that chromosome segregation in *Giardia* occurs through a spindle-independent mechanism (Solari et al., 2003). To determine conclusively whether dynamic microtubules are required for chromosome segregation, we treated mitotic cells with taxol [which binds to the β-tubulin subunit of the tubulin heterodimer in polymerized microtubules (Parness and Horwitz, 1981; Rao et al., 1995)] and evaluated segregation defects. Although giardial tubulin subunits are highly divergent, the β-tubulin subunit has retained the amino acid sequence associated with taxol binding (see supplementary material Fig. S1). Nearly 100% of mitotic cells treated with either 10 μM or 20 μM taxol (∼100) showed dramatic defects in chromosome segregation and...
spindle morphology (Fig. 5). In control cells treated with dimethyl sulfoxide (DMSO) abnormal cell divisions were rarely observed. Many cells had broken spindles with two half-spindles emerging from opposite spindle poles (Fig. 5C). Some elongated spindles were bent around the anterior edge of the cell with the chromatin lying outside the spindle axis (Fig. 5A,D).

In wild-type, untreated cells, anaphase A is completed in one nucleus before the other (data not shown). In cells treated with taxol, anaphase A was delayed and chromosome segregation occurred in both nuclei together (Fig. 5B). In some cases chromosome missegregation occurred in cells in the absence of any obvious spindle defects (Fig. 5B). In cells in which anaphase A segregation had taken place, the chromosomes did not remain tightly clustered at the poles (data not shown). Chromosome segregation defects ranged from one or two lagging chromosomes remaining in the middle of the dividing nucleus (Fig. 5C), to a trail of lagging chromosomes extending between the two spindle poles (Fig. 5D). Treatment with nocodazole also resulted in lagging chromosomes (data not shown).

Flagellar duplication
In some flagellates, such as Chlamydomonas, flagella are resorbed at the onset of mitosis and the basal bodies (as centrioles) are recruited to function as part of the mitotic spindle poles (Ou and Rattner, 2004). Both centrin localization and TEM data showed that while giardial flagellar basal bodies are present at the spindle poles, all eight flagella were retained during mitosis.

A dramatic rearrangement of the flagella coincided with nuclear migration in prophase; flagellar rearrangements were most easily seen with the anterior and caudal flagellar pairs. Migration of the nuclei to the center of the cell displaces the flagellar basal bodies. By TEM, we observed the central nuclei in an early mitotic cell separating the caudal flagellar pair (Fig. 6A). Movements of the anterior flagella were assessed by light microscopy. Basal bodies associated with each anterior flagellum moved to the outer periphery of the nuclei, associating with one of the four spindle poles (arrowheads in Fig. 6C,D). The fluorescence images of the anterior flagella matched the position of the flagella seen in longitudinal sections in TEM images of the spindle pole (Fig. 4B). These data confirm and extend a recent report of flagellar rearrangements during mitosis in Giardia (Nohynkova et al., 2006).

Discussion
Recent discourse has suggested that mitosis in Giardia proceeds by a unique and unprecedented mechanism (Benchimol, 2004a; Benchimol, 2004b; Solari et al., 2003). We find, however, that the major cytological events of mitosis in Giardia proceed as defined in many organisms: it begins with prophase condensation of chromatin, followed by the alignment of chromosomes in the spindle midzone, the movement of chromosomes to the spindle poles, the separation of spindle poles and the eventual duplication of other cellular structures prior to cytokinesis. Although we were unable to identify an aligned metaphase plate in giardial mitosis, which could be attributed to the transient nature of this stage or to the small size of the chromosomes, it is clear the chromosomes congress to the spindle midzone during metaphase. Both spindles are bipolar arrays of microtubules with attachments to chromosomes at kinetochores. Thus, the organization and function of the giardial spindle is highly conserved with these aspects of the metazoan spindle, despite the strikingly different morphology (i.e. two nuclei and eight flagella) and evolutionary distance between Giardia and metazoans.

Distinctive aspects of giardial mitosis (as compared with metazoan or fungal mitoses) include: the semi-open structure of the nuclear envelope and spindle; the movement of each nucleus to the cell center (one dorsal and one ventral); and the extended telophase spindle structure. Further, we infer some involvement of the flagellar basal bodies in spindle microtubule nucleation and/or spindle positioning (Fig. 4C). The unique features of Giardia mitosis are highlighted in detail below.
Spindle structure
Based on ultrastructural analyses, mitotic spindles in protists have been classified using several cytological features: the continuity of the nuclear envelope during mitosis (open, semi-open and closed); the position of the spindle relative to the nuclear envelope (pleuromitosis versus orthomitosis); and the position of chromosomes relative to the spindle axis (Raïkov, 1994). The nuclear envelope sequesters chromatin throughout most of the cell cycle. In most plants and animals, a complete nuclear envelope breakdown occurs during prophase, allowing microtubules direct access to chromatin. In protists, both kinetoplastids (e.g. trypanosomes) and heteroloboseans (e.g. *Naegleria gruberi*) retain an intact nuclear envelope, or closed mitosis, with an intranuclear spindle, a trait that is shared with both fission and budding yeast (O’Toole et al., 2003; Ogbadoyi et al., 2000; Schuster, 1975; Winey et al., 1995). The trichomonads also have a closed mitosis with an extranuclear spindle that attaches to kinetochores embedded in the nuclear envelope (Brugerolle, 1975b; Gomez-Conde et al., 2000).

During giardial mitosis, the nuclear envelope also acts as a barrier between cytoplasmic microtubule arrays and chromatin. By TEM, we provide the first evidence that *Giardia* has a semi-open mitosis in which microtubules from the two extranuclear spindles penetrate the nucleus through polar openings in the nuclear envelope (Fig. 4B). This is similar to a description of semi-open mitosis in a related diplomonad, *Hexamita inflata* (Brugerolle, 1975a), and is consistent with the diversity of mitotic spindle structure in protists. Owing to the ubiquity of extranuclear spindles in protists, the ancestral state of eukaryotic mitosis may be similar to *Giardia* and trichomonads, an extranuclear spindle that interacts with chromatin either through a semi-open nuclear envelope or across an intact nuclear envelope. In this scenario, both closed mitosis with intranuclear spindles (yeasts) and open mitosis (plants and metazoans) are derived mitotic forms.

Evidence for kinetochore microtubules in chromosome segregation
The mitotic spindle links the microtubule cytoskeleton to chromosomes through kinetochore microtubules to facilitate chromosome segregation. Two experimental observations strongly support the role of kinetochore microtubules in chromosome segregation in *Giardia*: the cenH3::GFP localization pattern in mitosis and the presence of intranuclear microtubules seen by TEM.

A histone H3 variant cenH3 is found exclusively at centromeres, and has been proposed as a universal marker of the eukaryotic centromere (Sullivan et al., 2001). Using a GFP-tagged cenH3 strain, we have shown that cenH3::GFP localizes to a discrete focus on each prophase chromosome, allowing us to visualize the giardial centromere and demonstrate that giardial chromosomes are monocentric (S.C.D. and W.Z.C., unpublished data). During prophase in other eukaryotes, centromeres containing cenH3 are required to build the mitotic kinetochore by recruiting motors, checkpoint proteins and additional structural elements (Van Hooser et al., 2001). In giardial mitosis, the clustering of cenH3-marked centromeres at the leading edge of segregating DNA (Fig. 2G) implies that chromosome segregation is mediated by microtubule attachments at the kinetochore.

The number of microtubules attached to the kinetochore ranges from a single kinetochore microtubule per chromosome in budding yeast to more than 20 per chromosome in some metazoans (McDonald et al., 1992; Winey et al., 1995). Although we have not directly observed kinetochore-microtubule associations, given the number of microtubules seen in a single TEM section and the number of kinetochores available for attachment in the nucleus, i.e. ten, we predict more than one microtubule is attached per kinetochore in *Giardia*.

Microtubule-stabilizing compounds generate lagging chromosomes on the anaphase spindle in *Giardia*. By inhibiting microtubule depolymerization using taxol, we were able to generate many of the same anaphase B spindle defects as previously observed in metazoans (Amin-Hanjani and Wadsworth, 1991; De Brabander et al., 1986; Jordan et al., 1993). Primarily, we found that taxol treatment caused the loss of interzonal microtubules, resulting in the formation of two half-spindles. This observation provides further evidence for the role of kinetochore microtubules in chromosome segregation and demonstrates the bipolar organization of spindle microtubules.

A role for the flagellar basal bodies in spindle organization and positioning
The morphology of the microtubule organizing centers...
(MTOCs) of mitotic spindles is diverse among eukaryotes. The centrosomes of metazoan cells consist of a pair of centrioles surrounded by pericentriolar material containing factors, such as gamma tubulin, required for microtubule nucleation (Ou and Rattner, 2004). Yeasts lack centrioles but are able to nucleate and organize the mitotic spindle using spindle pole bodies (SPBs) (Jaspersen and Winey, 2004). Flagellated protists such as *Chlamydomonas* recruit flagellar basal bodies, structurally related to centrioles, to function as part of the MTOC at the spindle poles following resorption of the flagella. These basal bodies of *Chlamydomonas* organize the spindle microtubules in a bipolar array and also appear to play a role in spindle positioning (Coss, 1974; Ehler et al., 1995). The association of flagellar basal bodies with the spindle poles may serve to ensure equal partitioning of basal bodies in each division cycle, thereby preserving the number of flagella in each daughter cell (Dutcher, 2003; Marshall and Rosenbaum, 2000; Heath, 1980; Lechtreck and Grunow, 1999; Wright et al., 1989).

In *Giardia*, eight basal bodies are inherited by each daughter cell during a mitotic division that includes two spindles and four spindle poles. How are the 16 giardial basal bodies accounted for during division and what roles do they play in spindle formation, positioning and cell-polarity determination? We hypothesize that the polarity of each daughter cell is established through the association of the eight basal bodies with the four spindle poles during mitosis. This proposal is based on our analysis of spindle structure using TEM, which demonstrates the association of multiple basal bodies with spindle poles (Fig. 4). We presume that one basal body at each pole acts as the central structural component of the MTOC (as indicated by the spindle microtubules radiating from the basal body seen in cross-section, see Fig. 4C). We predict that careful analysis of γ-tubulin in the intermediate stages of mitosis as outlined in our study would reveal γ-tubulin as part of the spindle-pole complex. In a recent study, γ-tubulin localization was detected at basal bodies both before and after nuclear division, although spindles were not directly observed (Nohynkova et al., 2000). We found that a second basal body is found at the periphery of the spindle-pole region, peripheral to the focused spindle microtubules (Fig. 4C). This basal body, with its associated flagellum, may play an indirect role in spindle nucleation and organization; its association with a spindle pole may ensure its proper segregation to the daughter cells. Our data support the recent description of movements of flagellar complexes during cell division (Nohynkova et al., 2006).

Outside of spindle nucleation, the association of flagellar basal bodies with spindle poles could establish and maintain cell polarity in each generation. We propose that specific combinations of two axonemes and associated basal bodies at each pole confer a unique positional identity to each of the four spindle poles. This is supported by both centrin immunostaining (Fig. 2K) and the TEM of telophase cells (Fig. 4D), which reveal flagellar basal bodies positioned between the two daughter nuclei on each side of the cell. The specific association of the eight basal bodies (with different developmental origins) with the four individual spindle poles can provide spatial cues for the proper establishment of cell polarity after mitosis.

**Nuclear repositioning, cytokinesis and the orientation of daughter cells**

The bilaterally symmetrical *Giardia* trophozoite is complex, and one would expect mitosis and cytokinesis to be tightly controlled and spatially defined processes. Based on our analysis of mitotic stages, we present a new model of giardial mitosis, the nuclear migration model, named for the repositioning of the two nuclei during prophase (Fig. 2C). In this model, the left and right nuclei are inherited with mirror image symmetry in the two daughter cells (Fig. 7). Although this pattern of nuclear inheritance has been predicted by previous studies (Benchimol, 2004a; Benchimol, 2004b; Čerčina and Nohynkova, 1992; Filice, 1952; Ghosh et al., 2001; Yu et al., 2002), the nuclear migration model differs in how the mirror image symmetry of daughter nuclei is achieved. To clarify the possible planes of cell division the axes of mitosis and cytokinesis are defined in Fig. 1C.

Three primary observations provide empirical support for our proposed model of mitosis: nuclear migration during prophase, cytokinesis perpendicular to the spindle axes and mirror image symmetry of nuclei using FISH. To illustrate this, we find that following prophase nuclear migration, nuclear division occurs along the left-right axis. The two nuclei remain separate during this time and thus do not exchange genetic information. Second, cytokinesis occurs in the longitudinal axis perpendicular to the axis of nuclear division.

Although it has been proposed that *Giardia* divides along multiple division planes depending on whether the cells are attached or unattached to a substrate (Benchimol, 2004a), this is not consistent with our observations. We examined populations of cells that were both attached and unattached at the time of fixation, and determined that chromosome segregation only occurs along the left-right axis of the cell with cytokinesis perpendicular to that plane. In the course of our experiments hundreds of mitotic cells were imaged and we never detected cytokinesis occurring in the dorsal-ventral plane.

Despite differences in terms of the timing and mechanism of cell division among diverse eukaryotes, the cell division plane is always perpendicular to the axis of chromosome segregation as defined by the mitotic spindle (Balasubramanian et al., 2004). Our model for cytokinesis, occurring in the longitudinal plane perpendicular to the spindle axis, is consistent with patterns of cytokinesis in other eukaryotes.

Finally, we show that nuclei are inherited with mirror image symmetry. In cytokinesis, we observed mirror image symmetry of nuclei containing the episomal FISH signal, as has been previously reported (Ghosh et al., 2001). Given that cytokinesis occurs in the longitudinal plane, the only outcome for nuclear identity is mirror image symmetry between the daughter cells.

Prior attempts to define the pattern of nuclear inheritance have also used FISH of an episomal plasmid to track the fate of either nucleus during cell division, but did not look at intermediate stages of mitosis, particularly during the prophase nuclear migration or the later four nuclear pre-cytokinesis stage (Ghosh et al., 2001; Yu et al., 2002). In addition, these studies presumed that cytokinesis occurs in the frontal plane or dorsal-ventral axis. Ghosh et al. reported that 50% of the FISH signal was present in either the left or right nucleus and in one nucleus per daughter in heart-shaped cells undergoing cytokinesis (Ghosh et al., 2001). Based on these observations, Ghosh and colleagues concluded that frontal cell division occurred, resulting in daughter cells in a ventral-ventral orientation. Alternatively, Yu et al. observed that the same nucleus always
Mitosis in *Giardia* contained the episomal plasmid, concluding that the cells were in a dorsal-ventral orientation (Yu et al., 2002). The pattern of FISH we observe supports the observations of Ghosh et al., wherein the two nuclei are inherited with mirror image symmetry. The fact that no cells contained two labeled nuclei in this or prior studies implies that: (1) there is no nuclear fusion during giardial mitosis; and (2) daughters inherit one copy of each parental nucleus in each generation.

Implications and possible mechanisms of the nuclear migration model for the maintenance of heterozygosity in the two nuclei

Beyond establishing the plane of cytokinesis, the mode of *Giardia* cell division has important implications that bear on the maintenance of a unique genetic identity for each nucleus. Based on our observations, the nuclei remain physically and genetically distinct from one another with daughter cells inheriting one copy of each parental nucleus in each generation. Has not been observed (Baruch et al., 1996; Kabnick and Peattie, 1990; Lu et al., 1998; Yu et al., 2002). The recent identification of putative meiotic genes in *Giardia* (Ramesh et al., 2005) has raised the possibility that rare nuclear fusions or meiotic events provide a mechanism for chromosomal recombination and reduction of such heterozygosity. Yet canonical meiosis, including the presence of characteristic cytological evidence such as a synaptonemal complex, has not been directly observed in *Giardia* trophozoites. One possibility is that two genetically disparate nuclei could be maintained in *Giardia*, if during rare points in the life cycle (i.e. encystation) the two nuclei could fuse and homogenize genetic material. Alternatively, the inheritance of genetic material from both nuclei may somehow be important for cell survival.

What is the mechanism of nuclear migration? Centrin, a calcium binding protein associated with basal bodies and centrosomes of eukaryotic cells (Salisbury, 1995), may represent a link between flagellar segregation and nuclear migration. In algal cells, centrin is a major component of the nuclear basal body connector, a fibrous network that physically links the basal bodies to the nucleus (Brugerolle and Mignot, 2004a).
2003; Marshall and Rosenbaum, 2000; Salisbury et al., 1988; Wright et al., 1989; Wright et al., 1985). In interphase, we observed centrin immunolocalization solely at basal bodies (Fig. 2) in contrast to prior studies (Belhadi, 1995; Meng et al., 1996). During mitosis, we determined that centrin localized only to basal bodies associated with the four spindle poles (Fig. 2). Although centrin is not associated with all eight basal bodies, centrin fibers may connect spindle pole-associated basal bodies with other basal bodies in preparation for segregation during mitosis. Thus, basal body migration and nuclear migration during mitosis may be coordinated events, facilitated by the centrin-dependent attachment of basal bodies to the nuclear envelope.

Conclusions

Diplomonads have been proposed to represent the earliest diverging lineage of extant eukaryotes, based on single rRNA and single and/or concatenated protein phylogenies using the best available phylogenetic methods when an archaeal outgroup is included (Baldauf, 2003; Baldauf et al., 2000; Ciccarelli et al., 2006; Van de Peer et al., 2000). In any evolutionary analysis, diplomons remain monophyletic and lack any statistically supported affiliation with late-evolving groups of eukaryotes or with the majority of so-called excavate taxa (with the possible exception of retortamonads or trichomonads) (Simpson, 2003; Simpson et al., 2006; Simpson et al., 2002).

Regardless of phylogenetic position, giardial mitosis shares common aspects of mitosis with other eukaryotes. In this way, insights into mitotic function in Giardia are informative for understanding general questions of spindle organization in diverse eukaryotes as compared with commonly studied experimental models. In terms of the evolution of the mitotic spindle, the observations of spindle organization in diplomons such as Giardia with the comparison with other protists leads to two main conclusions. First, mitosis in both Giardia and the trichomonads occurs with extranuclear spindles that interact with chromatin across the nuclear membrane. Therefore, this may represent a more ancestral state of mitotic organization than either the open mitosis of plants and metazoans or closed mitosis of fungi and trypanosomes. Second, monocentric chromosome structure is probably ancestral to holocentric chromosome structure.

At a minimum, understanding both the molecular conservation of structure and function of the dual spindles in such a highly divergent eukaryote provides a unique perspective on the mechanism of mitosis and its evolution. Intriguing prospects remain to be determined; in particular, how conserved is mitosis at the molecular level in Giardia? Also, how do we account for the observed homogeneity between the nuclei in populations of Giardia given our proposed nuclear migration model? In the long-term, a detailed understanding of both the mode and mechanism of giardial cell division will be crucial toward developing anti-giardial compounds that target the cell division machinery.

Materials and Methods

Conclusions

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experiments determining the contribution of the microtubule cytoskeleton to chromosome segregation, taxol was diluted directly into culture tubes containing media to a working concentration of either 10 μM or 20 μM. An equal concentration of DMSO was added to control cells.

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References


Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Olsen, G. J. and Bork, P. (2006). An equal concentration of taxol was diluted directly into culture tubes containing media to a working concentration of either 10 μM or 20 μM. An equal concentration of DMSO was added to control cells.

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Yu, L. Z., Birky, C. W., Jr and Adam, R. D. (2002). The two nuclei of giardia each have complete copies of the genome and are partitioned equatorially at cytokinesis. *Eukaryotic Cell* 1, 191-199.
Table S1. Percentage of mitotic cells in semi-synchronous cultures following the replacement of fresh medium at one-hour intervals

<table>
<thead>
<tr>
<th></th>
<th>Interphase</th>
<th>Mitotic (all stages)</th>
<th>Cytokinesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=524)</td>
<td>97%</td>
<td>1.3%</td>
<td>0.9%</td>
</tr>
<tr>
<td>3 hours (n=524)</td>
<td>92%</td>
<td>4.3%</td>
<td>3.4%</td>
</tr>
<tr>
<td>4 hours (n=547)</td>
<td>82%</td>
<td>11.8%</td>
<td>2.1%</td>
</tr>
<tr>
<td>5 hours (n=551)</td>
<td>92%</td>
<td>1.4%</td>
<td>5.6%</td>
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