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Segregation of a microsporidian parasite during host cell mitosis

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SUMMARY

We investigated the segregation of an intracellular microsporidian parasite during host cell division. A time-course experiment was carried out to examine the distribution of parasites relative to host chromosomal DNA via light and electron microscopy. Fluorescent light microscopy and EM studies showed that the parasite lay in the perinuclear zone of the host cell during interphase and segregated to daughter cells at mitosis. At metaphase, the parasite was frequently closely associated with host microtubules and mitochondria. Electron-dense bridges were observed between the parasites and the host microtubules and also between host mitochondria and microtubules. The study suggests that both the parasite and the host cell organelles segregate in association with spindle microtubules.

Key words: microsporidian, mitosis, microtubule, mitochondria, *Nosema* sp.

INTRODUCTION

We have previously described a vertically transmitted microsporidium (*Nosema* sp.) found in the crustacean host, *Gammarus duebeni* (Terry, Dunn & Smith, 1997). The parasite is one of a number of microsporidia which are transmitted via the oocyte and exert a feminizing influence on infected offspring, thus, in effect acting as cytoplasmic sex determining factors (Bulnheim & Vavra, 1968; Bulnheim, 1971; Dunn, Adams & Smith, 1993; Terry *et al*. 1997; Terry, Smith & Dunn, 1998). During early host embryogenesis parasite replication was slower than host cell division. Parasite numbers remained exceptionally low in juvenile hosts and in adults, where they were restricted to gonadal tissue (Terry *et al*. 1997). Throughout host development only intracellular merogonic stages were seen and we found no evidence of sporulation. This implies that the parasite reaches its target tissue via differential segregation through specific host cell lineages. Studies of parasite distribution in early host embryos lend support to this hypothesis (Dunn, Terry & Taneyhill, 1998). Two further questions arise, first, what is the cellular mechanism of parasite segregation and, secondly, how does this mechanism enable differential targeting of host tissue. In the current paper we address the first question through a detailed time-course experiment following the distribution of the parasite throughout host cell mitosis.

The mechanism of parasite segregation may resemble that of cytoplasmic organelles. The distribution of organelles, such as mitochondria and membrane-bound vesicles, during mitosis has been shown to be associated with the spindle apparatus, implying that segregation is mediated by the microtubular cytoskeleton (Daniels & Roth, 1964; Waterman-Storer, Sanger & Sanger, 1993). The association of mitochondria with microtubules is also supported by their overlapping distribution in interphase cells (Heggeness, Simon & Singer, 1978; Summerhayes, Wong & Chen, 1983), while direct evidence for microtubule-mediated movement comes from studies of saltatory motion (Aufderheide, 1977). Cross-bridges between organelles and microtubules have been visualized by electron microscopy of interphase cells (Allen, 1975; Vale, 1987) although not during mitotic division.

In previous light microscopy studies the microsporidian parasite has been observed to cluster in the region of the spindle poles of dividing host cells (Terry *et al*. 1997). To evaluate the role of the host cell microtubular cytoskeleton in parasite segregation we have monitored parasite distribution through mitosis via light microscopy and related this to parasite–organelle association in parallel EM studies.

MATERIALS AND METHODS

Thirty laboratory bred, female *G. duebeni* infected with *Nosema* sp. and 10 uninfected controls were paired with uninfected males in containers of 150 ml brackish water at 12 °C and allowed to mate (Dunn & Hatcher, 1997). Food included rotted leaves and marine green alga, *Enteromorpha* spp. Embryos (approximately 16 per brood) were flushed from the brood pouch of anaesthetized females within 4 h of fertilization and cultured in brackish water at 20 °C.

Embryos from 15 infected females and 5 controls

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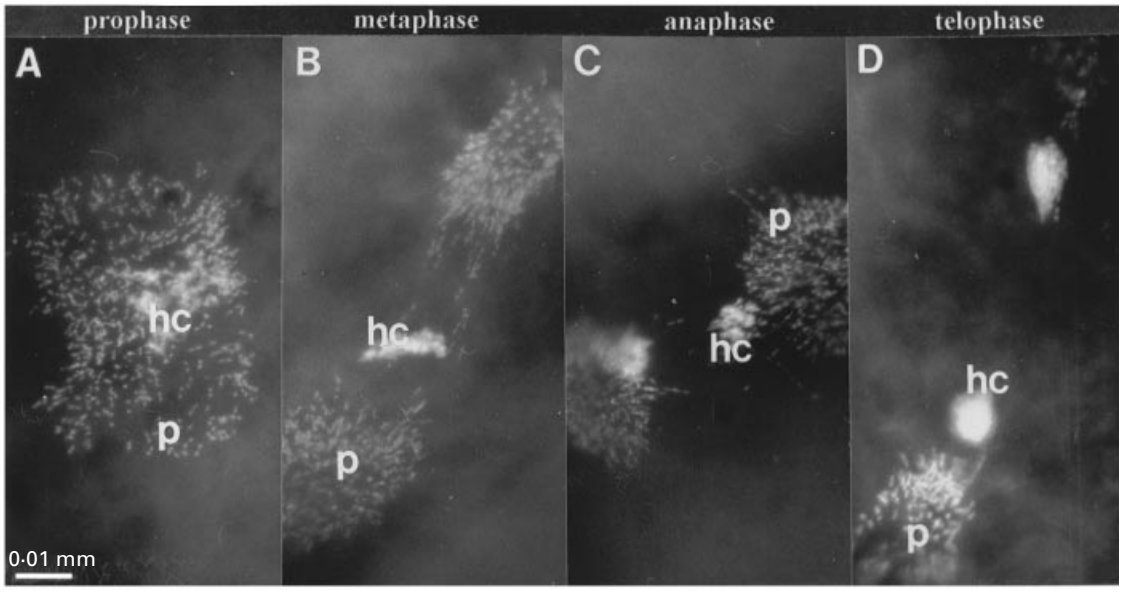

Fig. 1. Fluorescent light micrographs showing the distribution of DAPI-stained microsporidian parasites (p) relative to host chromosomes (hc) during the 8–16 cell host embryonic division. (A) During prophase (T_{5min}) the parasites are uniformly distributed around the condensing host chromosomes. (B) At metaphase (T_{15}) the host chromosomes are aligned on the spindle plate and the parasites are located in 2 distinct clusters at the opposite poles of the dividing nucleus. (C) During anaphase (T_{20}) the host chromosomes have moved towards the spindle poles while the parasites remain in this area. (D) At telophase (T_{25}) the parasites are associated with the 2 reorganizing daughter host nuclei.

were used in light microscopy (LM) studies. Division is normally synchronous up to the 16-cell stage and is also synchronous between embryos derived from the same brood. Each brood was observed at 15 min intervals and the timing of the first 3 divisions noted. To follow parasite distribution throughout mitosis, embryos were then taken at 5 min intervals from a point (T_0), approximately 30 min prior to the fourth division (8–16 cells), fixed and stained with DAPI (4,6-diamidino-2-phenyl-indole) a fluorescent dye for DNA (Terry *et al*. 1997). Embryos were screened using a Zeiss Axioplan fluorescent microscope. The stage of mitotic division was revealed by the position of host chromosomes and the position of parasites was recorded. From these data the timing of mitotic division was recorded.

Embryos from the remaining control and infected females were removed and observed to establish the timing of division as above. Embryos were then fixed during interphase (T_0) and metaphase (T_{15}) in 2·5 % glutaraldehyde and 6 % tannic acid in 0·1 M cacodylate buffer (pH 6·9) for 4–5 h. Specimens were then rinsed in 0·1 M cacodylate buffer, post-fixed overnight in 1 % osmium tetroxide, rinsed in buffer, dehydrated through a graded ethanol series and embedded in Araldite (Luft, 1961). Semi-thin and ultra-thin sections were cut through entire embryos using a glass knife on a Reichert–Jung ultracut microtome. Semi-thin sections were stained with toluidine blue (1 % in borax) to assess the structure of the tissue. Ultra-thin sections were taken through entire cells (diameter = 0·25 mm). They were stained with uranyl acetate and lead citrate and examined on a Jeol JEM-1200EX electron microscope. Images shown are representative of approximately 350 infected sections screened. Parasite–mitochondrial association was assessed by counts on 100 randomly selected parasites in interphase and metaphase cells. The diameter of mitochondria along the longest axis was taken as an index of mitochondrial size, using a random sample of 100 parasite-associated and free mitochondria in interphase and metaphase cells. All mitochondrial data were analysed statistically using *t*-tests.

RESULTS

Direct observation of *G. duebeni* embryos via light microscopy revealed that cell division was synchron-

FCR (*c*). (*D*) *In infected cells at metaphase parasites are restricted to the perinuclear cytoplasm* (*pc*) *at the spindle poles* (*hc = host chromosome*, *mt = microtubule*, *m = mitochondria*, *p = parasite*). (*E–I*) *Parasite–organelle interactions at metaphase* (T_{15}). (*E and F*) *Parasites are localized in areas of abundant microtubules and are frequently attached or closely juxtaposed to these host microtubules* (*mt*). (*G*) *The plasma membrane* (*pm*) *of the parasite is in close contact* (→) with the host microtubule. (H) Close contact (→) is seen between the outer membrane of the host mitochondria (m) and the host microtubule (mt). (I) Parasites (p) are in close association with host mitochondria (m). (J) The parasite (p) and host mitochondrion (m) are flattened together to form a junction (j).

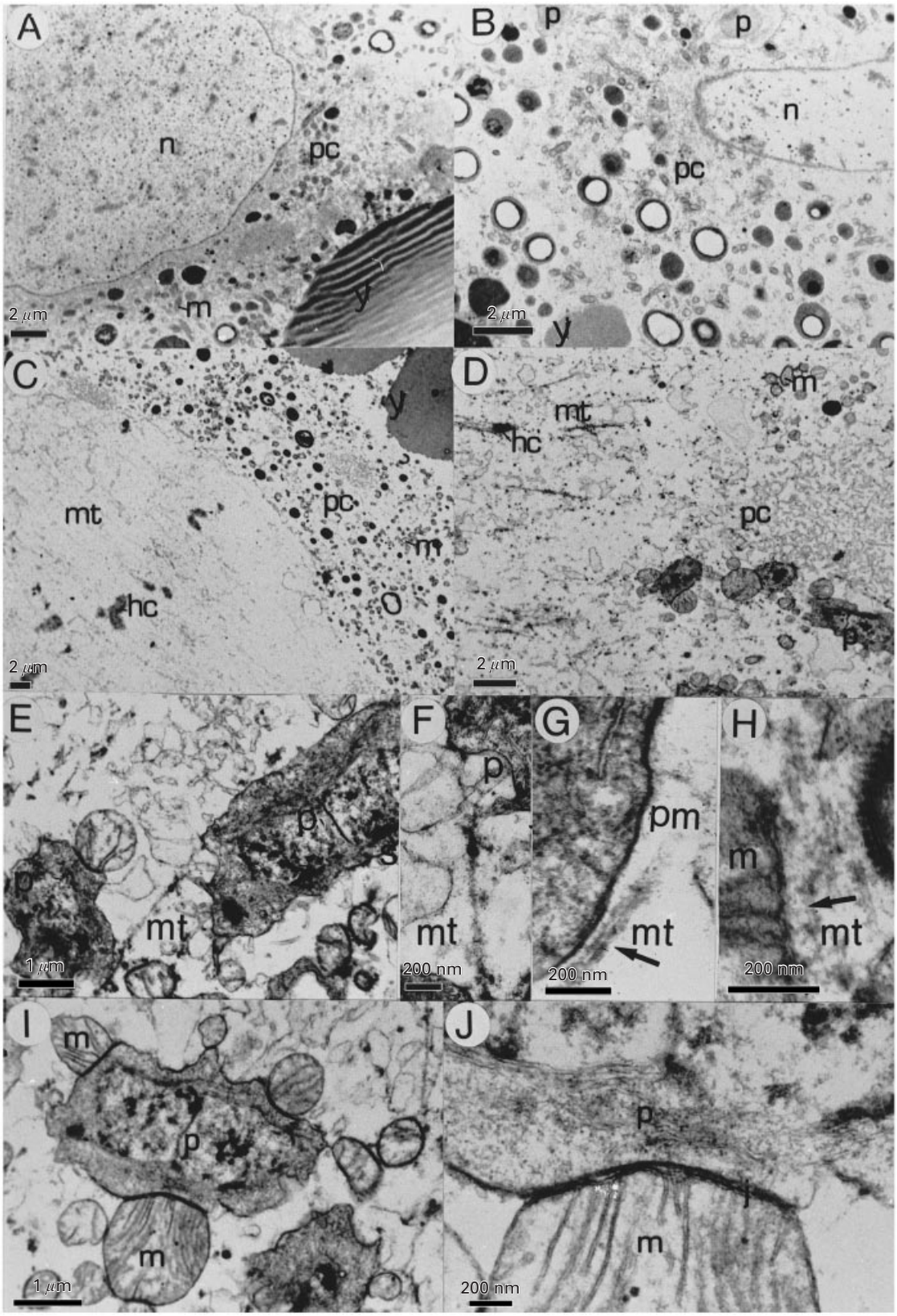

Fig. 2. Electron micrographs showing parasite distribution in host cells during interphase (T_0) and metaphase (T_{15}). (A) In uninfected cells at interphase the nucleus (n) is surrounded by a narrow band of perinuclear cytoplasm (pc) containing organelles such as mitochondria (m). This region is further surrounded by the yolk zone (y) containing numerous lipid globules. (B) In infected host cells at interphase parasites (p) can be seen within the perinuclear cytoplasm (pc). (C) In uninfected cells during metaphase the host chromosomes (hc) are aligned along the central axis of the spindle microtubules (mt). Organelles, such as mitochondria (m), are distributed in the perinuclear cytoplasm

[*for continuation see opposite*

Table 1. The number and size of host mitochondria attached to parasites

(The mean number ± S.E. of attached mitochondria per parasite and the mean size (μm) of these organelles is given at interphase and metaphase.)

Mitochondria	Interphase cells	Metaphase cells	Test
Number per parasite	2·11 ± 0·152	2·76 ± 0·823	$t_{193} = 3{\cdot}26$, $P < 0{\cdot}01$
Size	0·4 ± 0·06	0·8 ± 0·04	$t_{57} = 5{\cdot}70$, $P < 0{\cdot}01$

ous within broods collected from the same female. No difference was observed in the division rate of parasitized and unparasitized embryos and the first 4 divisions (1–16 cells) occurred at regular intervals of 120 min. Parallel observations of nuclear DNA, via DAPI staining, demonstrated that the transition from interphase to prophase occurred approximately 30 min prior to cleavage.

The distribution of parasites, relative to host cell nuclear DNA, during the process of host cell division was followed by DAPI staining. During interphase, parasites were evenly distributed around the host cell nucleus in the perinuclear cytoplasm. At the onset of mitosis, during prophase, the parasites remained around the host nucleus (Fig. 1A). At metaphase the host chromosomes were aligned on the spindle plate and the parasites began to form 2 discrete clusters (Fig. 1B). Through anaphase the axis of host cell division became more clearly defined and parasite clusters appeared to be associated with the spindle poles (Fig. 1C). During telophase the parasites were redistributed from their polar axes to surround the newly formed daughter nuclei (Fig. 1D).

The structure of the host cell was clearly visualized via electron microscopy. During interphase the nuclear region, containing chromatin, was surrounded by a nuclear envelope and then by an area of perinuclear cytoplasm, which contained organelles such as mitochondria but no yolk material (Fig. 2A). The remainder of the cell cytoplasm, outside this perinuclear zone, contained abundant yolk material in addition to organelles. Parasites were always found within the perinuclear cytoplasm of interphase cells (Fig. 2B). EM of cells in metaphase showed that, although the nuclear envelope had broken down, the 2 main nuclear zones and the peripheral cytoplasm were retained. The nuclear region could be defined as the area in which abundant spindle microtubules were found and this contained little other than ribosomes and chromosomes arranged along an equatorial plate (Fig. 2C). The perinuclear zone contained fewer microtubules, residual patches of the nuclear envelope, mitochondria and abundant endoplasmic reticulum. The basic structural organization of control and parasitized cells was identical. However, in parasitized cells, the perinuclear zone contained large numbers of microsporidian parasites (Fig. 2D).

In detailed examination of the perinuclear zone of metaphase cells, microtubules were often seen in parallel arrays radiating from the spindle towards the peripheral yolk zone. Scanning of sections taken through the entire host cell revealed that both parasites and their associated host cell mitochondria were exclusively localized in the area of these microtubules (Fig. 2E, F). At higher magnification both parasites (Fig. 2G) and host mitochondria (Fig. 2H) were frequently seen to be closely juxtaposed to or in contact with host microtubules.

In both interphase and metaphase cells, parasites were directly attached to mitochondria. The boundary between these two bodies was extremely close (Fig. 2I), with the outer mitochondrial membrane flattened against the parasite plasma membrane to form a margin that resembled a gap junction (Fig. 2J). The number of parasite-associated mitochondria increased from interphase to metaphase (Table 1). In addition, the mean diameter of the mitochondria which were attached to parasites, was larger than that of free mitochondria (mean diameter: attached mitochondria, 0·8 μm ± S.E. 0·04; unattached, 0·6 μm ± S.E. 0·3, $t_{197} = 3{\cdot}91$, $P < 0{\cdot}01$).

DISCUSSION

The association between microsporidia and host mitochondria has been well established (Sprague & Vernick, 1968; Canning, Okamura & Curry, 1997). Microsporidia do not possess mitochondria and rely on the host cell for their energy requirements (Canning & Lom, 1986). In interphase cells, we found that host mitochondria lay in close proximity to, and occasionally in contact with the parasites. A similar level of contact has been reported for other microsporidia in interphase host cells (Pell & Canning, 1993; Sprague & Vernick, 1968) but mitosis has not previously been studied. In this study we have described, for the first time, the association between parasites and host mitochondria during host cell mitosis. We found an increased area of contact between the parasite plasma membrane and the outer mitochondrial membrane. This zone of contact was exceptionally close, resembling a gap junction.

The size and number of mitochondria in contact with the parasite plasma membrane increased from

interphase to metaphase. This may reflect accumulation of mitochondria by the parasite during its growth cycle prior to division. We have previously established that parasite numbers increase from the 8–16 cell stage of embryogenesis onwards (Terry *et al*. 1997). Alternatively it is possible that parasites specifically associate with mitochondria during metaphase.

Visualization of the parasite through host cell division showed clearly that there was an association with the spindle apparatus external to the nucleoplasm. This may benefit both the parasite and its host. The parasite is provided with a means of segregation, whilst both host and parasite benefit from the exclusion of the parasite from the spindle zone, which ensures that the process of nuclear division is not disrupted.

The pattern of segregation of the microsporidium resembles that seen in previous studies of the partitioning of the mitochondria to the daughter cells (Daniels & Roth, 1964). Similarly, membranous organelles (Waterman-Storer *et al*. 1993), membrane-bound vesicles (Allen, 1975) and pigment granules (Kobayakawa, 1988) have all been shown to segregate along the axis of the spindle. This indirect evidence suggests that organelles are attached to and translocated relative to spindle microtubules. However, although cross bridges have been seen between organelles and microtubules during interphase (Smith, Jarlfors & Cayer, 1977), they have not been seen during mitosis.

In the current study we have seen both physical translocation of the parasite relative to the spindle during mitosis and direct evidence of parasite association with microtubules. In addition to this direct contact, parasites were attached to host cell mitochondria which in turn were associated with microtubules. This evidence provides strong support for microtubule-based segregation of the parasite. Similar links have been reported between other parasite species and the host cell cytoskeleton. *Theileria parva* is associated with host cell microtubules within the spindle during the division of bovine lymphocytes (Hulliger *et al*. 1964; Vickerman & Irvin, 1981). In addition, proteobacteria in *Drosophila* embryos have been shown to be linked to the astral microtubules through electron-dense bridges (Callaini, Riparbelli & Dallai, 1994).

Eukaryotic cell organelles are thought to have evolved from endosymbionts (Margulis, 1981) and, during this process, a mechanism for segregation at host cell division has developed. Our study suggests that the microsporidian parasite shares the same strategy for segregation at host cell division. It is interesting to question the nature of the molecular mechanism of microtubular attachment and whether this is also shared with organelles. The parasite may provide a useful tool to study the mechanisms of segregation and the evolution of endosymbiosis.

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