Mitosomes of Entamoeba histolytica are abundant mitochondrial-related remnant organelles that lack a detectable organellar genome

Gloria León-Avila and Jorge Tovar

School of Biological Sciences, Royal Holloway, University of London, Egham TW20 0EX, UK

The existence of mitochondrial-related relict organelles (mitosomes) in the amitochondrial human pathogen Entamoeba histolytica and the detection of extranuclear DNA-containing cytoplasmic structures (EhKOs) has led to the suggestion that a remnant genome from the original mitochondrial endosymbiont might have been retained in this organism. This study reports on the mutually exclusive distribution of Cpn60 and extranuclear DNA in E. histolytica and on the distribution of Cpn60-containing mitosomes in this parasite. In situ nick-translation coupled to immunofluorescence microscopy failed to detect the presence of DNA in mitosomes, either in fixed parasite trophozoites or in partially purified organellar fractions. These results indicate that a remnant organellar genome has not been retained in E. histolytica mitosomes and demonstrate unequivocally that EhKOs and mitosomes are distinct and unrelated cellular structures.

INTRODUCTION

Over the past few years mitochondrial remnant organelles (mitosomes) have been identified in a number of amitochondrial parasitic protozoa (e.g. Giardia intestinalis, Entamoeba histolytica, Trachipleistophora hominis and Cryptosporidium parvum) (Mai et al., 1999; Tovar et al., 1999, 2003; Williams et al., 2002; Riordan et al., 2003), disproving the Archezoa hypothesis, which postulated that amitochondrial eukaryotic organisms were the direct descendants of the nucleated cell that hosted the original mitochondrial endosymbiont (Cavalier-Smith, 1983, 1998). It is now apparent that eukaryotic microbes that lack typical mitochondria are not primitive amitochondrial but are highly derived descendants of mitochondrial-containing ancestors whose capacity for aerobic respiration has been gradually lost during the course of evolution.

Because of their recent discovery, little is known about the physiological functions of mitosomes. Some, such as those of Entamoeba and Cryptosporidium, have been shown to contain chaperonin Cpn60, a protein known to participate in the refolding of imported proteins (Sigler et al., 1998; Mai et al., 1999; Tovar et al., 1999; Riordan et al., 2003); mitosomes of Trachipleistophora contain a mitochondrial-type Hsp70 (mtHsp70), a molecular motor that helps internalize proteins into the organelle (Matouschek et al., 2000; Williams et al., 2002). Other proteins have also been suggested as putative mitosomal components in various amitochondrial lineages but their cellular localization has not been demonstrated experimentally (Clark & Roger, 1995; Bakatselou et al., 2000, 2003; Katinka et al., 2001; Morrison et al., 2001; Zhu & Keithly, 2002; Arisue et al., 2002). Perhaps the most significant finding in relation to the biology of mitosomes is the direct demonstration that Giardia mitosomes function in the biosynthesis of molecular iron–sulphur (Fe–S) clusters and in their subsequent incorporation into functional Fe–S proteins (Tovar et al., 2003). Genes encoding several proteins involved in Fe–S cluster metabolism have also been identified in the genomes of several other amitochondrial organisms, including Encephalitozoon cuniculi, Entamoeba histolytica, C. parvum and Trichomonas vaginalis (Katinka et al., 2001; Tacheye et al., 2001; LaGier et al., 2003; Bankier et al., 2003; http://www.sanger.ac.uk/Projects/E_histolytica/ and http://www.tigr.org/tdb/e2k1/eha1), leading to the suggestion that the requirement for Fe–S proteins probably represents the selective pressure driving the retention of the original mitochondrial endosymbiont in all eukaryotic lineages (Tovar et al., 2003; van der Giezen & Tovar, 2004; Embley et al., 2003b).

The presence of a remnant organellar genome with prokaryotic phylogenetic affinity is perhaps the strongest evidence for the bacterial ancestry of mitochondria. But whether E. histolytica mitosomes have retained an ancestral organellar genome has been a matter of debate and controversy. Mai et al. (1999) and Tovar et al. (1999) found no evidence for the presence of DNA in Cpn60-containing mitosomes (syn. cryptons) using Hoechst dye and propidium iodide staining, respectively. Further studies...
by Ghosh et al. (2000), however, suggested the existence in cryptons of a large organellar genome estimated at 2-2 % of the nuclear genome (about 880 kb in size). This finding fuelled speculation that mitosomes/cryptons might be the same organelle as the DNA-containing cytoplasmic structures described by Orozco and collaborators in *E. histolytica* – termed kinetoplast-like organelles (EhKOs) (Orozco et al., 1997). Without mounting evidence for the autogenous nature of EhKOs (i.e. originating from an internal cellular compartment as opposed to having an external endosymbiotic origin) (Orozco et al., 1997; Rodriguez et al., 1998; Luna-Arias et al., 1999; Solis et al., 2002; Marchat et al., 2003; Mendoza et al., 2003), the question of whether or not the DNA-containing EhKOs could be the same as the Cpn60-containing mitosomes has remained unresolved.

Here, we have used laser scanning confocal microscopy and a specific antibody against *E. histolytica* Cpn60 to show that mitosomes are abundant organelles present in all parasite trophozoites and that their distribution is different from that of the DNA-containing EhKOs, which are only present in a subset of the parasite population. We also use one of the most powerful molecular techniques for the detection of DNA – *in situ* nick-translation coupled to immunofluorescence microscopy – to demonstrate that the mitosomes of *E. histolytica* lack a detectable organellar genome.

**METHODS**

**Parasite culture.** *E. histolytica* trophozoites were cultured axenically in YI-S medium with 15 % adult bovine serum as described by Clark & Diamond (2002).

**DNA detection.** *E. histolytica* trophozoites were detached from culture tubes by incubation on ice and collected by centrifugation at 275 g for 10 min. Cells were washed in PBS, fixed in 4 % paraformaldehyde in PBS for 30 min at 37 °C and then washed and resuspended in 1 ml PBS. Fixed cells were attached to polylysine-coated glass slides, washed twice in PBS and permeabilized with 0-2 % Triton X-100 at room temperature for 5 min. Following permeabilization, slides were rinsed twice in PBS and subjected to *in situ* nick-translation using biotinylated dATP and a Nick-Translation System (Invitrogen) as described by the manufacturer. Reactions were incubated for 4 h at room temperature and washed four times, blocked with 1 % BSA in PBS and incubated for 1 h at 37 °C with streptavidin–FITC conjugate diluted 1:200 (v/v) in blocking buffer.

**Mitochondria selective stain.** A 48 h monolayer of mouse muscle cells C2C12 (ATCC, Manassas, VA, USA), growing in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % FCS, was treated with 0-25 % trypsin in EDT buffer (136 mM NaCl, 50 mM KCl, 50 mM EDTA, 25 mM Tris pH 7-5) for 2 min, detached by resuspension, centrifuged at 500 g for 10 min and resuspended in 1-2 ml fresh DMEM complete medium. Cells (8·9 x 10^6) were applied on each glass slide and attached to the surface for 3 h at 37 °C in a 5 % CO2 incubator. Following removal of medium and addition of 200 µl fresh medium containing 125 mM MitoTracker Red (Molecular Probes), slides were incubated at 37 °C for 30 min, washed twice in PBS and fixed in 4 % paraformaldehyde for 30 min at 37 °C. Cells were permeabilized with 0-2 % Triton X-100 at room temperature for 5 min and subjected to *in situ* nick-translation as described above.

**Immunodetection of Cpn60.** *E. histolytica* slides were blocked with 1 % BSA in PBS (blocking buffer) for 30 min and incubated with specific rabbit anti-Cpn60 antibodies overnight at 4 °C; samples were washed repeatedly in PBS and further incubated with a goat anti-rabbit TRITC conjugate (Pierce) diluted 1:300 in blocking buffer.

**Purification of microsomal fraction.** Cell fractionation was done according to Tovar et al. (1999), with some modification. The cells were lysed by freeze–thawing and the microsomal fraction was obtained by centrifugation at 100,000 g for 30 min at 4 °C. Aliquots were fixed in 4 % paraformaldehyde in PBS, attached to glass slides and permeabilized by treatment with acetone for 5 min at room temperature. After repeated rinsing in PBS, fractions were subjected to *in situ* nick-translation and anti-Cpn60 treatment as described above.

**Laser scanning confocal microscopy.** After treatments, all slides were washed five times in PBS and mounted with 15 µl VectaShield mounting medium (Vector). Slides were observed under a scanning confocal microscope (Radiance 2100; Bio-Rad) fitted with Argon and HeNe laser beams. Images were collected using the Bio-Rad LASersharp 2000 software and processed with POWERPOINT version 7.0.

**RESULTS**

Laser scanning confocal immunomicroscopy of fixed *E. histolytica* trophozoites treated with antibodies raised against recombinant Cpn60 from the parasite (recEhCpn60) show the compartmentalized distribution of the protein in discrete foci distributed throughout the cytoplasm (Fig. 1). Confocal optical slices of 500 nm thickness revealed that Cpn60-containing mitosomes are smaller and more abundant than previously estimated by low-resolution fluorescence microscopy (Tovar et al., 1999). The number of mitosomes in the trophozoites shown in Fig. 1(a) was 256 as estimated by counting labelled structures in stacks of confocal optical slices of 500 nm thickness. Most organelles were only observed in single optical slices, but some labelled structures could be seen over two and occasionally three confocal slices. Maximum projection images showed considerable overlap of label due to the random distribution of organelles throughout the cell cytoplasm. All trophozoites in the parasite populations analysed displayed Cpn60-containing mitosomes.

The putative presence of a remnant organelar genome in *E. histolytica* mitosomes was investigated using one of the most powerful techniques currently available for the detection of DNA, *in situ* nick-translation coupled to immunofluorescence microscopy (Clemens & Johnson, 2000). Nuclear DNA was readily detected in all trophozoites analysed, whereas extranuclear DNA was only detected in a minority percentage of the cell population (Fig. 2). Immunodetection of Cpn60 in these cells confirmed the presence of multiple mitosomes in each cell of the parasite population but no co-localization of Cpn60 and DNA label was detected. Computer-assisted magnification of mitosomes and of DNA-containing structures in merged images further demonstrated that the cellular distribution of Cpn60 and of extranuclear DNA in *E. histolytica* is mutually exclusive (Fig. 2b). To test the limits of detection of DNA by *in situ*
nick-translation we used mammalian muscle cells as a control. The 16·3 kb mitochondrial genome of mouse cells (Bayona-Bafaluy et al., 2003) was readily detected by confocal microscopy (Fig. 2c), demonstrating that an organellar genome as small as 16·3 kb in size can be readily identified using this technique.

To further investigate the presence of extranuclear DNA in *E. histolytica*, we prepared a mixed-membrane subcellular fraction enriched in biological membranes and membrane-bounded organelles. Immunofluorescence microscopy of the mixed-membrane fraction, incubated with anti-Cpn60 antibodies and subjected to *de novo* DNA biosynthesis by *in situ* nick-translation, revealed the presence of DNA-associated and Cpn60-containing subcellular structures/membranes (Fig. 3). However, no co-localization of Cpn60 and DNA labelling was detected, confirming that the cellular distribution of Cpn60 and DNA is mutually exclusive in this parasite.

**DISCUSSION**

The Archezoa hypothesis, now abandoned by its original proposer (Cavalier-Smith, 2002), crumbled under the weight of evidence for the presence of relict mitochondrial organelles in several organisms representative of the major amitochondrial eukaryotic lineages (Archeamoïda, Metamonada, Parabasalia and Microsporidia), rejecting the idea that those organisms diverged from the main eukaryotic trunk prior to the endosymbiotic acquisition of the mitochondrion.

This study provides evidence that *E. histolytica* mitosomes are abundant mitochondrion-related organelles that lack a detectable organellar genome. High-resolution confocal microscopy revealed the presence of dozens of Cpn60-containing mitosomes in every single parasite trophozoite. The fact that most organelles were only seen in single optical slices limits their size to a maximum of 500 nm (the thickness of the optical slice). Larger labelled structures spanning two or occasionally three optical slices observed in some cells indicate the presence of either closely associated mitosomes not resolved by the methodology employed or of individual structures of 1·5 μm maximum diameter. These are likely to represent the structures previously observed by low-resolution fluorescence microscopy (Tovar et al., 1999).

The mutually exclusive distribution of Cpn60 and DNA in confocal optical slices of parasite trophozoites demonstrates unequivocally that the Cpn60-containing mitosomes and the DNA-containing EhlKOs are distinct intracellular entities of different provenance. *De novo* DNA biosynthesis and Cpn60 labelling of partially purified membrane-bounded organelles confirmed the mutually exclusive nature of Cpn60-containing and DNA-associated cell structures. Detection of biotinylated DNA in the mixed-membrane fraction indicates the presence of cellular structures that contain, or are associated with, DNA; these structures can be taken as a positive control for the detection – given its presence – of a putative organellar genome in Cpn60-containing mitosomes. The lack of a detectable mitosomal genome is consistent with the absence of a
detectable organellar genome in the mitochondrion-related hydrogenosomes of *Trichomonas* (Dyall & Johnson, 2000; Clemens & Johnson, 2000; Embley *et al*., 2003a). Although our failure to detect DNA in mitosomes does not formally exclude the theoretical existence of a very small mitosomal genome, it is clear that such remnant genome would have to be significantly smaller than 16 kb to escape detection using this methodology, as the 16·3 kb mouse mitochondrial genome was readily detected in control experiments (Clemens & Johnson, 2000; Bayona-Bafaluy *et al*., 2003). In this respect, the reported existence of a large 880 kb organellar genome in the Hsp60-containing crypton (syn. mitosome) is puzzling (Ghosh *et al*., 2000).

Our data suggest that the genome of the original mitochondrial endosymbiont in *E. histolytica* mitosomes has been lost – like many other mitochondrial components – through reductive evolution. Most genes present in the genome of the original mitochondrial endosymbiont have been transferred to the cell nucleus. Biosynthesis of nuclear-encoded organellar proteins is thus carried out in the cytoplasm, with subsequent targeting of these proteins into their respective organelles through highly sophisticated protein import mechanisms (Hauke & Schatz, 1997; Kunau *et al*., 2001). It has recently been hypothesized that the reason why all known mitochondria (and chloroplasts) have retained a highly reduced but functional genome might be

Fig. 2. Detection of DNA in *E. histolytica* and in control mammalian cells by in situ nick-translation. Fixed *E. histolytica* trophozoites treated with anti-Cpn60 antibodies and fixed muscle cells pre-treated with MitoTracker Red were subjected to DNA synthesis by in situ nick-translation using biotinylated dATP; de novo synthesized DNA was detected using streptavidin–FITC and confocal microscopy. Panels depicting individual and merged confocal images are presented. (a) Single parasite trophozoite representative of cells where no extranuclear DNA is observed (>65% of the cell population). (b) Single trophozoite representative of cells where extranuclear DNA is detected (<35% of the cell population). Insets on the merged image show computer-assisted magnification of indicated organelles highlighting the lack of co-localization between DNA and Cpn60. (c) Control mouse muscle cells showing mitochondrial and nuclear genomes.
that – as most of the remaining genes encode the structural proteins responsible for the maintenance of the redox balance in the cell – the expression of redox proteins needs to be tightly regulated on site to avoid the potential toxic effects of their electron transfer activities during ATP biosynthesis (Allen & Raven, 1996; Race et al., 1999; Allen, 2003). It is proposed that such tight regulation requires the co-location of genes and gene products for efficient operation (Allen, 2003). In this respect, the fact that ATP biosynthesis is now longer compartmentalized in E. histolytica but occurs via substrate level phosphorylation in the cell cytosol is highly relevant (Müller, 2003). The selective pressure for the retention of a mitosomal genome would have eased as the organelle was freed from the constraints of electron transfer activities, a relaxation that may have led to the complete loss of the mitosomal genome.

All known endosymbiosis-derived organelles, i.e. mitochondria, chloroplasts, mitosomes and hydrogenosomes, are self-replicating entities that segregate faithfully into each daughter cell following asexual cell division. In contrast, the proportion of the parasite population harbouring EhKOs appears to be cell-cycle-dependent, with as few as 10 and 20 % of the population displaying these structures at G2 and S phases, respectively (Rodríguez et al., 1998). In experiments with unsynchronized parasite trophozoites the percentage of the cell population harbouring extranuclear DNA does not exceed 50 % (Orozco et al., 1997; Rodríguez et al., 1998; Luna-Arias et al., 1999), a distribution not consistent with that of an essential cellular organelle carrying out vital cellular functions. EhKOs have been shown to emanate from the cell nucleus, to be cell-cycle-regulated and to contain eukaryotic-type rRNA, a pyruvate:ferredoxin oxidoreductase-like protein and a number of eukaryotic-type transcriptional regulators, including a TATA box-binding protein, an enhancer-binding protein and a tumour-suppressor protein (Orozco et al., 1997; Rodríguez et al., 1998; Luna-Arias et al., 1999; Solis et al., 2002; Marchat et al., 2003; Mendoza et al., 2003). The presence of eukaryotic-type nuclear components and the recent demonstration that EhKOs emanate from the cell nucleus confirm the autogenous nuclear origin of EhKOs and invalidate the suggestion that these cellular structures might be self-replicating organelles of endosymbiotic origin. The apparent biogenesis of minute vesicles from the cell nucleus in the diplomonad G. intestinalis (Benchimol, 2002) – akin to the process observed in E. histolytica (Solis et al., 2002) – betrays the existence of an enigmatic biological phenomenon which may be unique to these parasites and which deserves further investigation. Given the different origins, cellular distribution and molecular composition of mitosomes and EhKOs, we conclude that mitosomes and EhKOs of E. histolytica are distinct and unrelated subcellular structures.

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REFERENCES


