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Minireview

Entamoeba histolytica mitosomes: Organelles in search of a function

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Abstract

It has been more than eight years since the discovery of mitosomes (mitochondrial remnant organelles) in the intestinal human pathogen *Entamoeba histolytica*. Despite detailed knowledge about the biochemistry of this parasite and the completion of the *E. histolytica* genome sequencing project no physiological function has yet been unequivocally assigned to these organelles. *Entamoeba* mitosomes seem to be the most degenerate of all endosymbiosis-derived organelles studied to date. They do not appear to participate in energy metabolism and may have dispensed completely with the proteins required for iron–sulphur cluster biosynthesis. However, the large number of mitosomes found in *E. histolytica* trophozoites hints at a significant biological role for these organelles in their natural environment. Identifying the protein complement of mitosomes will provide answers as to their biological significance and the reason(s) for their retention in this parasite.

Index Descriptors and Abbreviations: Amoeba; Anaerobic metabolism; ATP transporter; Chaperone; *Cryptosporidium*; *Dictyostelium*; Energy metabolism; *Entamoeba*; *Giardia*; Hydrogenosome; Iron–sulphur; *Mastigamoeba*; Mitochondria; Mitosome; Organelle; Protist; Protein import; Protozoa; Pyruvate oxidation; *Trichomonas*; ATP, adenosine triphosphate; Cpn60, chaperonin 60; FMN, flavin mononucleotide; Isc, iron–sulphur cluster; *luc*, firefly luciferase gene; μm, micrometer; mtHsp70, mitochondrial-type Hsp70; NAD, nicotine adenine dinucleotide; NADP, nicotine adenine dinucleotide phosphate; NifS, cysteine desulphurase; NifU, iron-binding scaffold protein; NO_3, nitrate; PFO, pyruvate ferredoxin oxidoreductase; PNT, pyridine nucleotide transhydrogenase; rRNA, ribosomal ribonucleic acid

1. Introduction

Mitosomes are mitochondrion-related organelles found in a range of unicellular eukaryotic organisms that inhabit oxygen-poor environments. Although dissimilar in appearance from text-book mitochondria, mitosomes harbour a small number of mitochondrial marker proteins and are surrounded by a double membrane. These observations have been interpreted as evidence that mitosomes represent degenerate mitochondria and as a result these organelles are considered excellent models for the study of mitochondrial evolution.

Mitochondria are responsible for the efficient preservation of chemical energy in the form of ATP which allows the cell to carry out the myriad of biological functions required for its survival. All multicellular organisms possess mitochondria which in general can be classified into two different types: aerobic and anaerobic mitochondria. Aerobic mitochondria are the most widely studied energy-generating organelles – they utilise oxygen as the final acceptor of electrons during aerobic respiration. Anaerobic mitochondria are present in organisms that spend at least part of their life cycles under conditions of oxygen deprivation (anaerobiosis) and contain the biochemistry required to utilise organic (e.g., fumarate) or inorganic (e.g., NO_3) compounds as final acceptors of electrons during anaerobic respiration (Tielens et al., 2002; Risgaard-Petersen et al., 2006). The evolution of different types of aerobic and anaerobic mitochondria is thought to have been driven by selective pressures specific to diverse

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environmental niches colonised by mitochondrion-containing organisms. Regardless of their diversity in form and function, a vast volume of experimental evidence supports the monophyletic nature of mitochondria (Gray et al., 1999).

In addition to the more visible multicellular eukaryotes, there is a large collection of nucleus-containing unicellular organisms representing a wide spectrum of eukaryotic diversity. Like their multicellular descendents, all aerobic microbial eukaryotes contain aerobic forms of mitochondria. However, anaerobic microbial organisms have traditionally posed a major challenge to biologists for two reasons: (i) sampling is usually problematic because they live mostly in anaerobic ecological niches that are difficult to access without sophisticated equipment (e.g., lake and ocean beds) or without disrupting their natural environment (in vertebrate and invertebrate digestive tracts) and (ii) they are either fastidious to grow or not amenable to axenic cultivation under laboratory conditions, making them difficult biological models for biochemical and genetic study. Traditional ultrastructural studies of anaerobic microbial organisms (e.g., *Entamoeba, Giardia*) consistently failed to identify cellular structures that could be construed as mitochondria. As a result, anaerobic microbial eukaryotes were for many years thought to be primitively amitochondrial – i.e., protoeukaryotic cells with nuclei hypothesised to have existed prior to the endosymbiotic acquisition of mitochondria – and to derive their biological energy exclusively by fermentation.

Over the past few years however it has become apparent that this interpretation is incorrect. All those anaerobic microbial eukaryotes studied in sufficient detail so far have been found to contain mitochondrion-related organelles known as hydrogenosomes or mitosomes, depending on whether or not they evolve molecular hydrogen as a product of their metabolism. The discovery of mitochondrion-related organelles in these microbial organisms has provided biologists with much needed biological models to investigate the evolutionary origins of mitochondria and the early evolution of the eukaryotic cell. Comparative studies aimed at identifying morphological, physiological and biochemical differences/similarities between mitosomes, hydrogenosomes, aerobic and anaerobic mitochondria are currently in progress in many laboratories worldwide. These studies are fuelled by genome wide comparative surveys in the postgenomic era. Experimental data emanating from such investigations have challenged traditional views of eukaryogenesis and are informing the design of alternative testable hypotheses to explain the origins of the eukaryotic cell.

Over the past few years the rate of progress in the field has been unprecedented. Several review articles and at least two books have been written recently about the biology and evolutionary significance of mitochondrion-related organelles and their contribution to our understanding of the early evolution of the eukaryotic cell (Embley et al., 2003; Williams and Keeling, 2003; Hirt and Horner, 2004; van der Giezen and Tovar, 2005; van der Giezen et al., 2005b; Embley, 2006; Embley and Martin, 2006; Martin and Müller, 2007). The interested reader will find in those references a wider and more in depth exploration of this area of study. In this minireview we will focus attention on what is known about the biology of *Entamoeba histolytica* mitosomes with comparative reference to mitosomes and hydrogenosomes from other parasitic protists where appropriate.

2. *Entamoeba* phylogeny and mitosome discovery

Entamoeba histolytica is an intestinal parasite of humans that colonises the large intestine. Although most infections are asymptomatic a small proportion (~10%) of infected individuals develop amoebic colitis as a direct result of *E. histolytica* infection (Stanley, 2003). Under conditions that are not yet fully understood the parasite may occasionally penetrate the intestinal wall and reach internal organs, particularly the liver, causing abscesses that can be fatal if untreated. During its life cycle the parasite undergoes cyclical morphological transformations between a colonising trophozoite and an environmentally resistant infective cyst, a process of differentiation that is essential for parasite infectivity and disease transmission. Of the various *Entamoeba* species known to colonise the human intestine, namely *Entamoeba dispar, Entamoeba coli, Entamoeba histolytica, Entamoeba moshkovskii, Entamoeba polecki* and *Entamoeba chattoni,* only *E. histolytica* has been unambiguously shown to cause symptoms of disease. *Entamoeba* species however are closely related. Phylogenetic analysis of ribosomal RNA gene sequences has demonstrated the monophyletic nature of the *Entamoeba* genus (Silberman et al., 1999). Initially no free-living amoebas appeared to be related to the lineage leading to *Entamoeba* but it is now known through multigene phylogenetic analysis that *Mastigamoeba* and *Dictyostelium*, two free-living amoebas, are relatives of *Entamoeba* (Bapteste et al., 2002). As a result, the amoebid lineages Entamoebidae (represented by *Entamoeba*), Mastigamoebidae (represented by *Mastigamoeba*) and Eumycetozoa (represented by *Dictyostelium*) are now grouped together in the Cluster Amoebozoa (Adl et al., 2005).

An early phylogenetic tree based on the small subunit ribosomal RNA genes found *Entamoeba* to be a postmitochondrial branch, diverging from the main eukaryotic trunk well after mitochondrion-containing lineages such as Euglenoids and Kinetoplastida (Sogin, 1991). Although the early and late branches of this tree were subsequently shown to be the result of methodological artifact (Gribaldo and Philippe, 2002), this finding was taken as evidence that the absence of recognisable mitochondria in *Entamoeba* was likely due to organelle decay or loss and not to primitive absence. Support for this view came from the work of Clark and Roger (1995) who amplified and cloned from the *Entamoeba* genome two genes encoding mitochondrial marker proteins, chaperonin 60 (Cpn60) and pyridine

nucleotide transhydrogenase (PNT). Phylogenetic analysis of Cpn60 using parsimony, distance and maximum likelihood methods demonstrated that the *Entamoeba* Cpn60 clustered with strong support with mitochondrial homologues, to the exclusion of bacterial sequences (Clark and Roger, 1995). Subsequent independent characterisation of *E. histolytica* Cpn60 in two laboratories led to the demonstration that Cpn60 is indeed expressed in trophozoites. Although disagreement exists as to whether or not the protein is upregulated upon heat shock, Cpn60 was found to be compartmentalised in small cytoplasmic structures that were named mitosomes (related to mitochondria; Tovar et al., 1999) or cryptons (cryptic function; Mai et al., 1999). The term mitosome is now widely used in the field to refer to mitochondrion-related organelles that have lost their capacity to synthesise ATP.

3. Mitosome morphology

The discovery of mitosomes in *Entamoeba* has been followed by the identification of mitosomes in various other "amitochondrial" microbial organisms across the taxonomic range, amongst others the microsporidian *Trachipleistophora hominis*, the alveolate *Cryptosporidium parvum* and the diplomonad *Giardia intestinalis* (Williams et al., 2002; Riordan et al., 2003; Tovar et al., 2003; Putignani et al., 2004). The morphology of *E. histolytica* mitosomes has not been unequivocally established by immunoelectron microscopy but enriched organelle preparations used in transmission electron microscopy experiments demonstrated the presence of small organelles of around half a micron in diameter surrounded by double membranes (Ghosh et al., 2000). Size-restricted confocal imaging of trophozoites incubated with a specific homologous anti-Cpn60 antibody revealed the presence of dozens of Cpn60-containing structures no bigger than 0.5 μm distributed throughout the cytosol (León-Avila and Tovar, 2004). Mitosomes from other organisms are also tiny (0.2–0.5 μm in *Cryptospridium* and <0.2 μm in *Giardia* and *Trachipleistophora*; (Williams et al., 2002; Tovar et al., 2003; Putignani et al., 2004; Slapeta and Keithly, 2004). Their minute sizes provide an explanation why – in the absence of molecular markers – mitosomes have been consistently overlooked in traditional transmission electron microscopy studies.

4. Mitosome biochemistry

To date the only protein directly demonstrated by immunomicroscopy to be present in *E. histolytica* mitosomes is Cpn60 but mtHsp70, Hsp10 and an unusual ATP/ADP transporter have all been shown to co-localise with Cpn60 in mitosome purification experiments (Chan et al., 2005; van der Giezen et al., 2005a; Tovar et al., 2007). None of these typical mitochondrial proteins reveal by themselves much about the potential function(s) of *E. histolytica* mitosomes. The biochemistry of *Entamoeba* was extensively investigated during the 1950s–1970s and with the completion of the *Entamoeba* genome project most of the metabolic pathways identified or predicted to exist in the early investigations have now been confirmed (Reeves, 1984; McLaughlin and Aley, 1985; Avron and Chayen, 1988; Loftus et al., 2005). From these studies, some insight into the biochemistry of mitosomes has been gained.

4.1. Pyruvate metabolism

Although fermentative glucose catabolism in *Entamoeba* is by the classical Embden–Meyerhoff glycolytic pathway some of its enzymes rely on inorganic pyrophosphate instead of ATP for catalysis (Reeves, 1984; Loftus et al., 2005). In the absence of pyruvate dehydrogenase and of tricarboxylic acid cycle enzymes in this parasite, pyruvate is decarboxylated via pyruvate ferredoxin oxidoreductase (PFO), with subsequent energy-conserving formation of acetate through acetyl-CoA synthase (ADP-forming). Partial purification of PFO and acetyl-CoA synthase from the soluble fraction of *E. histolytica* extracts suggests that pyruvate metabolism is not compartmentalised in this parasite (Reeves et al., 1977). Furthermore, neither PFO nor acetyl-CoA synthase were found to sediment under conditions of ultracentifugation sufficient for mitosome sedimentation (>80,000×*g* for 30 min; Reeves et al., 1977; Tovar et al., 1999). That no significant PFO enzymatic activity has ever been demonstrated in mixed membrane fractions of *E. histolytica* urges caution over the interpretation of a controversial immunomicroscopy study that found a cross-reacting PFO-like antigen on the surface of *E. histolytica* trophozoites (Rodríguez et al., 1998).

4.2. Respiration

Under microaerophilic conditions of growth *Entamoeba* consumes oxygen without the generation of peroxide but it is unclear whether the gas is indeed used as the final acceptor of the electrons generated during pyruvate oxidation. It is also unclear how such electrons are transferred to NAD under anaerobiosis. Experimental evidence indicate that in the absence of heme iron-containing cytochromes, soluble NADPH diaphorase and/or NADPH:flavin oxidoreductase activities could transfer electrons released during pyruvate oxidation onto the iron–sulphur centres of ferredoxin via one or more flavins (e.g., FMN). The electron transfer steps after ferredoxin are unknown (Reeves, 1984; McLaughlin and Aley, 1985; Avron and Chayen, 1988; Loftus et al., 2005). All these proteins are found in the soluble fraction of *Entamoeba* extracts suggesting that electron transfer is not compartmentalised (Reeves, 1984; McLaughlin and Aley, 1985; Avron and Chayen, 1988; Loftus et al., 2005). PNT, the mitochondrial enzyme responsible for transhydrogenation from NADH to NADPH, has been associated with *E. histolytica* membranes but its precise cellular localisation has not been

unequivocally determined (Yu and Samuelson, 1994; Clark and Roger, 1995).

4.3. Iron sulphur cluster biosynthesis

In the absence of mitochondrial functions such as pyruvate oxidation, Krebs cycle, cytochrome-mediated electron transport, oxidative phosphorylation, haeme biosynthesis, fatty acid β-oxidation and the urea cycle, what might the function of *E. histolytica* mitosomes be? One of the most recently discovered functions of mitochondria is the biosynthesis of iron–sulphur (FeS) centres essential for the functionality of enzymes and electron transport proteins collectively known as FeS proteins (Lill and Muhlenhoff, 2006). To date FeS cluster assembly is the only biosynthetic function of mitochondria essential for cell survival under conditions of aerobic and anaerobic growth. Importantly, FeS cluster biosynthesis has also been identified as a function of *Giardia* mitosomes and of trichomonad hydrogenosomes (Tovar et al., 2003; Sutak et al., 2004). It has been hypothesised that FeS cluster biosynthesis provides the selective force behind the retention in eukaryotic cells of the original mitochondrial endosymbiont in the form of mitochondria, mitosomes and hydrogenosomes (Embley et al., 2003; Tovar et al., 2003).

Of special note is the fact that *E. histolytica* is the only eukaryote in which the presence of a bacterial type rather than a mitochondrial type FeS cluster assembly system has been reported (Ali et al., 2004; van der Giezen et al., 2004). Genes encoding two essential components of the nitrogen fixation (Nif) FeS cluster assembly system – cysteine desulphurase (NifS) and the iron-binding scaffold protein NifU – were identified in the genome of *E. histolytica*. Phylogenetic analyses clearly indicated that these proteins are not mitochondrial in origin but were acquired by the parasite by lateral gene transfer from epsilon-proteobacteria. The fact that *E. histolytica* possesses a non-redundant bacterial type FeS cluster assembly system suggests that the iron–sulphur cluster (Isc) system contributed by the original mitochondrial endosymbiont was replaced by the bacterial Nif system during the course of evolution. Unpublished data presented at recent conferences (e.g., Giselle Walker (2007) Meeting Report: 16th Meeting of the International Society for Evolutionary Protistology; Wroław, Poland, August 2–5, 2006 (ISEP XVI), Protist 158: 5–19) suggest that the free-living ''amitochondrial'' amoeba *Mastigamoeba* also harbours bacterial type FeS cluster assembly proteins and contains double membrane bounded mitosomes. If confirmed, these data would suggest that the replacement of the mitochondrial type Isc by the bacterial type Nif FeS cluster assembly system occurred in a common ancestor of *Entamoeba* and *Mastigamoeba*, before the adaptation of *E. histolytica* to parasitism.

An important question is whether or not FeS cluster assembly might have been retained in these organisms as a mitosomal function. The unequivocal intracellular localisation of NifS and NifU has not yet been reported but since neither NifS nor NifU contain putative aminoterminal targeting peptides that could target them into mitosomes their intracellular localisation might be cytosolic. On the other hand some bacterial proteins show natural predisposition for mitochondrial targeting so NifS and NifU may yet be found in mitosomes (Lucattini et al., 2004). Indirect support for this possibility comes from the observation that several mitosomal proteins in other mitosome-containing organisms such as *Giardia* and *Trachipleistophora* lack any detectable organelle targeting signals and yet are efficiently imported into mitosomes (Tachezy et al., 2001; Williams et al., 2002; Tovar et al., 2003; Regoes et al., 2005). Work is in progress in different laboratories worldwide to determine whether or not bacterial type FeS cluster assembly is a function of mitosomes.

5. Protein import

Most luminal mitochondrial and hydrogenosomal proteins are synthesised on cytosolic ribosomes and are imported into their respective organelles via their positively charged amino-terminal peptides. The first indication that *Entamoeba* Cpn60 and PNT might be imported into what was then a hypothetical organelle came from the realisation that their amino-terminal peptides were, like those of luminal mitochondrial proteins, rich in hydroxylated and basic amino acid residues (Clark and Roger, 1995). The hypothesis that these might function as organelle targeting signals was tested using deletion mutagenesis and cell fractionation of transgenic parasites. While wild type Cpn60 accumulated in the mixed membrane fraction (containing broken membranes and membrane-bounded organelles), a mutant version of Cpn60 lacking amino acids 2–15 was shown to accumulate in the cytosol. Such mutant phenotype was readily reversed by addition to the mutant protein of a functional mitochondrial targeting signal from trypanosomes (Tovar et al., 1999). These experiments demonstrated the requirement of the amino-terminal peptide for organelle targeting and provided the first evidence for the functional conservation of protein import mechanisms of *E. histolytica* mitosomes and mitochondria. Since then, the functional conservation of mitosomal and mitochondrial protein import has also been shown for a number of mitosomal proteins from *Giardia* and *Cryptosporidium*, including giardial ferredoxin and IscU as well as cryptosporidial Cpn60, mtHsp70, IscS and IscU (LaGier et al., 2003; Riordan et al., 2003; Tovar et al., 2003; Slapeta and Keithly, 2004; Dolezal et al., 2005; Regoes et al., 2005).

In an attempt to quantify the dynamics of protein import into *E. histolytica* mitosomes we made recombinant constructs encoding three variants of Cpn60-firefly luciferase fusion proteins: (i) the full-length protein, (ii) mutant Cpn60 lacking amino acids 2–15 and (iii) mutant Cpn60 lacking amino acids 66–536. Recombinant parasite lines carrying these constructs were fractionated by differential centrifugation and luciferase activity measured in their

mixed membrane and cytosolic fractions to quantify the distribution of reporter enzyme (Fig. 1). Parasites transfected with the full-length fusion protein deliver most of the protein into the mixed membrane fraction while those devoid of amino acids 2–15 displayed increased cytosolic localisation of the reporter protein. These data suggest that the amino terminal peptide of Cpn60, although required, is not sufficient for full organelle targeting under the experimental conditions used. Further, the observation that the initial 66 amino acids of Cpn60 deliver only a small proportion of the reporter protein into mitosomes suggests that other cryptic sequences within the Cpn60 protein may act synergistically in organelle targeting. At present it is unclear whether an amino-terminal targeting peptide is a universal requirement of protein import into *Entamoeba* mitosomes. In other mitosome-containing organisms such as *Giardia*, presequence-dependent and presequence-independent pathways of mitosomal protein import are operational (Dolezal et al., 2005; Regoes et al., 2005; Tovar, 2007).

To test whether the amino-terminal region of PNT – another putative mitosomal protein – could deliver a passenger protein into mitosomes we generated an expression construct that fused the initial 67 amino acids of PNT to firefly luciferase (Fig. 1e). Most of the luciferase activity of transgenic parasites was associated with the mixed membrane fraction suggesting that this sequence is sufficient for membrane targeting. However, because the aminoterminal sequence used contains two hydrophobic alpha-helices that could anchor luciferase into virtually any biological membrane, protease protection experiments were conducted to test whether the recombinant protein could be in the lumen of mitosomes – as would be predicted from the topology of mitochondrial PNT (Yamaguchi and Hatefi, 1991; Yu and Samuelson, 1994). Results presented in Table 1 shows that the membrane-associated luciferase was equally susceptible to trypsin degradation when compared to cytosolic luciferase from control parasites, suggesting that the reporter protein is likely embedded in plasma or ER membranes rather than in the inner membrane of mitosomes. Such artificial experimental system cannot of course rule out the possibility that native *E. histolytica* PNT might indeed reside in mitosomes. In this respect, the identification of PNT in the proteome of enriched preparations of *E. histolytica* phagosomes is equally inconclusive because the possibility of contamination with other organelles cannot be excluded (Okada et al., 2005,2006).

Table 1
Protease protection of recombinant firefly luciferase

	Luciferase activity (RLU)[a]	
	Eh 5′PNT-luc	Eh-luc
Untreated control	8382	6700
Triton X-100	8671	7470
Trypsin	114	8
Triton + trypsin	4	1
Triton + trypsin + trypsin inhibitor	9243	8040

[a] Total luciferase activity (in relative light units) in cell-free extracts from indicated parasite lines treated with 0.2% Triton X-100 and/or 0.1 mg/ml trypsin. Trypsin digestion of samples was allowed to proceed for 30 min on ice. A ten fold molar excess of trypsin inhibitor was used where indicated.

Considering the apparent functional conservation between protein import into *Trypanosoma cruzi* mitochondria and *E. histolytica* mitosomes an educated guess is that homologues of mitochondrial protein translocases might exist in *E. histolytica*. Despite extensive searchings, only a putative homologue of TOM40 has been identified in the *E. histolytica* genome (Loftus et al., 2005; van der Giezen et al., 2005b). It may be that the structure of mitosomal protein translocases has decayed beyond the point of recognition by current bioinformatics algorithms. Perhaps simpler membrane-bound translocators equivalent to those described for trichomonad hydrogensomes may operate in *E. histolytica* mitosomes (Carlton et al., 2007). Proteomic analysis of these organelles may help elucidate the nature of mitosomal translocases in *E. histolytica*.

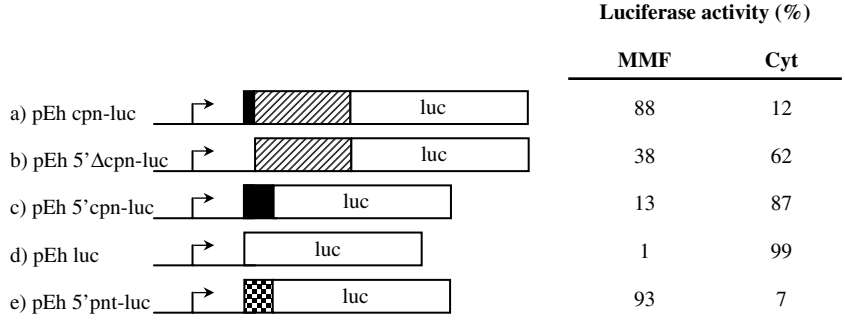

Fig. 1. Functionality of Cpn60 and PNT amino-terminal sequences in organelle protein import. Firefly luciferase activity from *E. histolytica* trophozoites transfected with chimaeric expression vectors containing segments of either *cpn60* (a–c) or *pnt* (e) fused to the *luc* reporter gene was quantified using a luminometer. The structure of recombinant plasmids is shown on the left. The cellular distribution of luciferase activity was determined following fractionation of parasite extracts by differential centrifugation. The mixed membrane fraction (MMF) contains broken cellular membranes and membrane-bounded organelles; non-sedimentable material remains in the cytosol (Cyt) after centrifugation. Luciferase activity is given as percentage of the total activity found in cytosol and membrane fractions. Each recombinant parasite line was tested at least twice in separate dates with similar results. Black boxes, 5′ *cpn60* region coding for amino acids 1–15 in pEhcpn-luc or aminoacids 1–66 in pEh5′cpn-luc; hatched boxes, cpn60 region coding for amino acids 16–536; chequered box, 5′ *pnt* region coding for amino acids 1–67; arrow, site of transcription initiation.

6. Mitosomal genome

One of the most sensitive and efficient methods to detect the presence of DNA in cells and tissues is *in situ*-nick translation coupled to immunofluorescence microscopy. This method has been used to look for the presence of a remnant organellar genome in *E. histolytica* mitosomes and in trichomonad hydrogenosomes but in neither case could such DNA be detected (Clemens and Johnson, 2000; León-Avila and Tovar, 2004). Moreover, no evidence of organellar DNA was obtained from the genome sequencing project (Loftus et al., 2005) further supporting the view that *Entamoeba* mitosomes lost their organellar genome through reductive evolution.

7. Conclusions and outlook

From the information presented above it is clear that *E. histolytica* mitosomes are remnants of the original mitochondrial endosymbiont in an organism adapted to a microaerophilic environmental niche. At present it is unclear what biological function(s) these organelles might fulfill but considering the large number of mitosomes present in all trophozoites of a parasite population it is reasonable to hypothesise that there must be at least one essential cellular function that drives organelle retention in *Entamoeba*. Currently the most likely candidate is FeS cluster metabolism. Given the availability of homologous antibodies against NifS and NifU the issue of their cellular localisation is likely to be resolved in the near future (Ali et al., 2004). Positive identification of these proteins in mitosomes would confirm that *E. histolytica* mitosomes, like those of *Giardia*, function in FeS cluster assembly; a negative result would leave these organelles still in search of a function. Proteomic studies of enriched mitosomal fractions will help identify the protein complement that make up these self-replicating but highly degenerate organelles and will shed light on the mechanisms of protein import and mitosome replication and inheritance. Perhaps the major challenge will come when attempting to assign a physiological role to mitosomal proteins identified in proteomics studies which are found to have no homologues in other organisms. Exciting times lie ahead in the search for the biological significance of *E. histolytica* mitosomes. Collectively, studies on the biology of mitosomes from different anaerobic microbial organisms will continue to inform our efforts towards understanding the evolutionary history of eukaryotes and of their endosymbiosis-derived organelles.

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