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Distinct Localization Patterns of Two Putative Mitochondrial Proteins in the Microsporidian *Encephalitozoon cuniculi*

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ABSTRACT. Microsporidia were once considered amitochondriate, but have now been found to retain relict mitochondria called mitosomes. These organelles have been identified by immunolocalization in *Trachipleistophora hominis*, whereas most data on function have been inferred from the presence of mitochondrial protein-encoding sequences in the genome of *Encephalitozoon cuniculi*. Here we describe the localization of two such enzymes in *E. cuniculi* cells. Immunofluorescent localization of ferredoxin involved in mitochondrial iron–sulfur cluster assembly reveals a punctate distribution as expected for mitochondria. In contrast, localization of mitochondrial glycerol-3-phosphate dehydrogenase suggests a cytoplasmic distribution in *E. cuniculi* and possible relocalization of this typically mitochondrial protein.

Key Words. Amitochondriate, *Encephalitozoon*, microsporidia, mitochondria, mitosome.

MICROSPORIDIA are obligate intracellular parasites that are common opportunistic infections of humans in the late stages of AIDS (Didier and Weiss 2006). Once thought to be primitive amitochondriate eukaryotes (Cavalier-Smith 1987), they are now widely accepted to be highly derived fungi or relatives of the fungi (Edlind et al. 1996; Keeling, Luker, and Palmer 2000). The mitochondria of microsporidia are highly reduced and are now among the most biochemically and physically diminished of mitochondria (Katinka et al. 2001). The organelle has been suggested to occur in several species of microsporidia on the basis of ultrastructural data (Vávra 2005) but so far has only been identified in one species using immunolocalization, *Trachipleistophora hominis* (Williams et al. 2002). In contrast, most of what is currently known about the probable functions of the organelle is based on the presence of several genes in the *E. cuniculi* genome coding for typically mitochondrial proteins (Katinka et al. 2001), but to date the location of none of these has been experimentally determined, and indeed the organelle has not actually been identified in this species. Proteins hypothesized to be targeted to a putative mitosome in *E. cuniculi* include several involved in organelle biogenesis, and several incomplete pathways, for example, a glycerol phosphate shuttle that does not lead into an electron transport chain and the E1 components of the pyruvate dehydrogenase complex, but not E2 or E3 components (Keeling and Fast 2002). The major hypothesized function of the *E. cuniculi* mitosome is inferred from the presence of several genes encoding proteins involved in the synthesis and cytosolic export of iron–sulfur clusters, which are essential mitochondrial functions in yeast (Lill and Kispal 2000). Iron–sulfur cluster assembly is also a common function of reduced mitochondria in other parasites, for example *Giardia* and *Trichomonas* (Tachezy, Sánchez, and Müller 2001). A second possible function for the *E. cuniculi* mitosome is the oxidation of NADH to sustain glycolysis in the cytosol via the glycerol-3-phosphate shuttle (Katinka et al. 2001).

Here we have immunolocalized homologues of two mitochondrial proteins in *E. cuniculi* that are involved in these two different processes. The first is ferredoxin, which is both an iron–sulfur cluster protein itself and also has an essential role in electron transfer in mitochondrial iron–sulfur cluster assembly (Lange et al. 2000). The second is FAD-dependent glycerol-3-phosphate dehydrogenase (GPDH), which is normally indirectly involved in ATP synthesis as the mitochondrial component of the shuttle that moves reducing equivalents across the mitochondrial membrane and into the electron transport chain (Ronnow and Kielland-Brandt 1993). It can also serve to reoxidise NADH to sustain glycolysis, and it is this role that the shuttle plays in other parasitic organisms such as *Trypanosoma brucei* and potentially *Cryptosporidium parvum* (Królová et al. 2000; Roberts et al. 2004).

MATERIALS AND METHODS

Rabbit polyclonal antibodies had been prepared commercially (AbCam, Cambridge, UK) against a mixture of two synthetic peptides from each protein. These peptide sequences were CHVILEEPLYRKLGE and CATKNMAVDGFKPKPH for the *E. cuniculi* ferredoxin protein, and CKMIEKPSEDWEPASR and CEKRHRGERRLPPQEK for the *E. cuniculi* mitochondrial GPDH. In both cases, the peptide sequences are non-conserved areas of the proteins and therefore exist only in these specific *E. cuniculi* proteins. Each antibody was affinity purified against the peptide sequences used to inoculate the rabbits. Infected rabbit kidney cells (RK-13 from American Type Culture Collection) were grown on cover slips until confluent and then fixed in 4% (w/v) paraformaldehyde, 1 × PBS (phosphate buffered salts) for 15 min and rinsed 3 times for 5 min in 1 × PBS. Cells were permeabilized in 0.1% (v/v) Triton X-100, 1 × PBS for 15 min. Cells were blocked with 1% (w/v) BSA (bovine serum albumin) solution and incubated overnight with a 1:200 dilution of anti-mitochondrial GPDH or anti-ferredoxin in 0.1% Triton X-100, 1 × PBS, 0.5% BSA. Slides were rinsed 3 times with 1 × PBS then incubated with a secondary anti-rabbit IgG Fluoronanogold Alexa 488 (Nanoprobes) for 1 h, rinsed, and mounted in a Dabco/Mowiol/DAPI solution. The labeled cells were visualized using an Axioplan Zeiss fluorescence microscope or using a Zeiss 510 meta confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA).

RESULTS AND DISCUSSION

Localisation of ferredoxin in *Encephalitozoon cuniculi*. Confocal and fluorescence microscopy revealed that ferredoxin was consistently localized to discrete structures within the *E. cuniculi* meront cells (Fig. 1–3). Using 13 confocal Z-stacks of 0.35 μm of an *E. cuniculi* parasitophorous vesicle containing 30 parasites, we counted the number of mitosome-like areas in each cell (Fig. 3). Here we found that most cells contained between two and four mitosomes (with one exceptional cell containing eight

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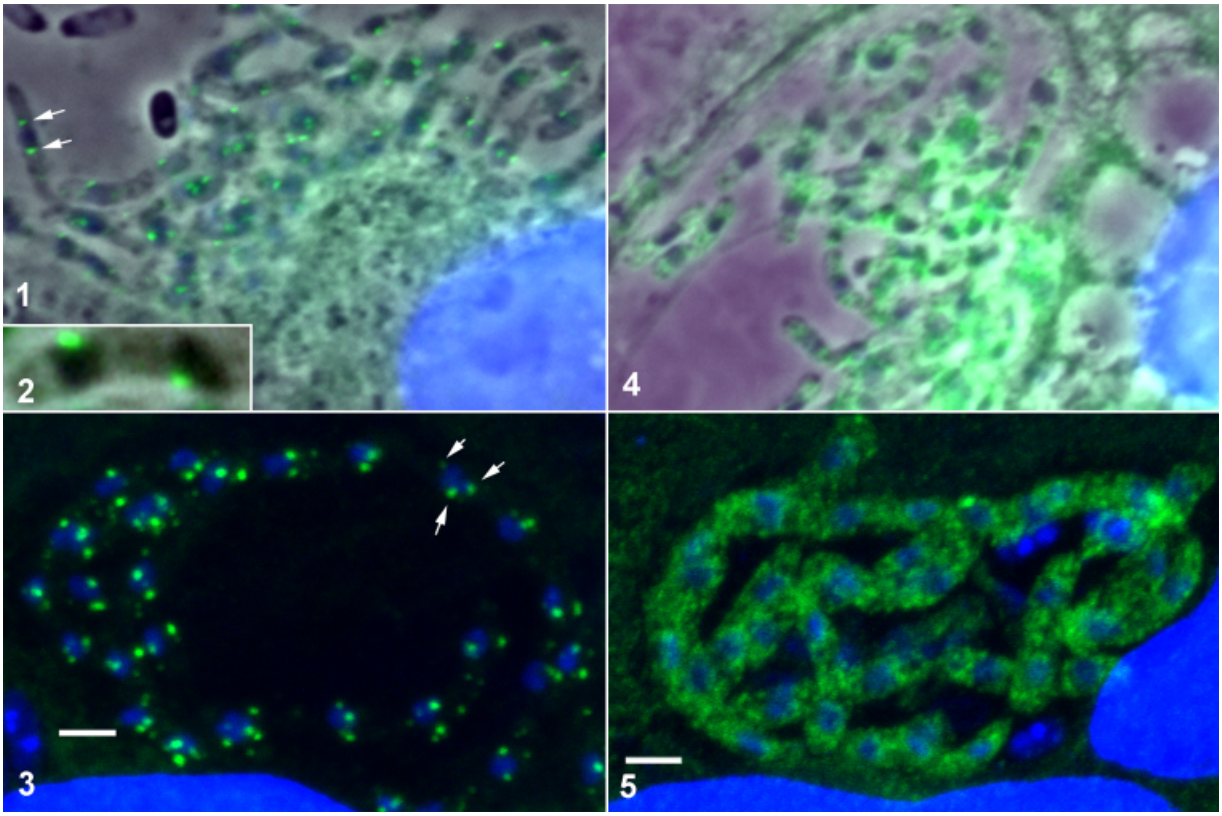

Fig. **1–5.** Localisation of ferredoxin (**1–3**) and mitochondrial glycerol-3-phosphate dehydrogenase (**4, 5**) proteins in *Encephalitozoon cuniculi* grown in RK-13 cells. **1.** Fluorescence image with the cell nuclei stained with DAPI and the anti-ferredoxin antibody pattern indicated in green. Mitosomes are indicated by white arrows. **2.** Two recently divided cells with a single mitosome next to the nucleus. **3.** A confocal projection of 13 confocal sections of 0.35 μm of a parasitophorous vesicle filled with *E. cuniculi* cells stained with DAPI and the anti-ferredoxin antibody. Again, mitosomes are indicated by white arrows. **4.** Fluorescence image with the cell nuclei stained with DAPI and the anti-mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) antibody pattern indicated in green. **5.** A confocal projection of 10 confocal sections of 0.35 μm of a parasitophorous vesicle filled with *E. cuniculi* cells stained with DAPI and the anti-mitochondrial GPDH antibody. Scale bars = 2 μm.

structures), with a mean, mode, and median number of 3 per cell. This is far fewer than the 7–47 mitosomes per cell previously observed by confocal microscopy in *T. hominis* (Williams et al. 2002). Interestingly, the distribution of mitosomes in the cell also differs from the random distribution seen in *T. hominis*. In the majority of cells, two mitosomes were consistently observed to flank the nucleus, while a third was situated in an irregular position in the cytoplasm (Fig. 1, 3). The two organelles flanking the nucleus are consistent with the position of ''polar vesicles,'' membrane-bounded structures observed in several microsporidia that have recently been proposed to be mitosomes based on their ultrastructure (Vávra 2005). We also observed meronts with a single nucleus-associated mitosome (Fig. 2). This association with the nucleus may exist to ensure that mitosomes are segregated to both daughter cells in dividing meronts (Vávra 2005).

Localization of GPDH in *Encephalitozoon cuniculi*. Antibodies specific for the *E. cuniculi* mitochondrial GPDH protein are not localized to discrete areas within the cell, but rather appear to be distributed throughout the cell, though not in a completely homogeneous distribution (Fig. 4, 5). This suggests that mitochondrial GPDH, unlike ferredoxin, is not specifically localized to the mitosome but may have taken on a new location in the cytosol sometime during the evolution of *E. cuniculi*.

The localization of ferredoxin to the *E. cuniculi* mitosome has revealed the number and position of the organelle in this species for the first time, and more importantly confirms that iron–sulfur cluster assembly biochemistry is most likely occurring within a discrete mitosome-like organelle in this species. However, the localization pattern for mitochondrial GPDH does not appear to be consistent with a mitosomal location protein, and therefore suggests the glycerol-3-phosphate shuttle is not functioning across the *E. cuniculi* mitosome membrane. This is of interest because the targeting properties of this protein have been shown to differ between two species of microsporidia. In heterologous targeting experiments the *E. cuniculi* protein was shown to be targeted to the vacuole of the *S. cerevisiae* cell while the *A. locustae* protein was recognized and taken up by the *S. cerevisiae* mitochondrion (Burri et al. 2006). Interestingly, mitochondrial GPDH appears to be processed in *A. locustae* spore proteins, whereas *E. cuniculi* lacks a processing enzyme and the native protein appears to be unprocessed in spores (Burri et al. 2006). The *E. cuniculi* genome also lacks a clear candidate for a mitosomal oxidase, which would be necessary for GPDH to function in a shuttle to move reducing potential to the mitosome, for example to recycle NAD^+ for glycolysis. All available data from localization, heterologous targeting experiments, examination of protein processing in spores, and functional inferences from genome data are therefore consistent with the conclusion that the glycerol-3-phosphate shuttle may no longer be a function of the *E. cuniculi* mitosome.

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